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13. ABSTRACT *(Maximum 200 words)*

The aim of this project is the development of reaction centers from photosynthetic bacteria for use in molecule-based components and devices. Reaction centers (RCs) exhibit a highly efficient, rapid, long-distance photoinduced charge separation under a wide variety of conditions. The reaction center has electronic properties which are far superior, more flexible, and much less expensive to produce than any known synthetic donor/acceptor system. During the course of this project we developed strategies for the attachment of reaction centers to electrode surfaces, and demonstrated the first steps in this process. Our approach employed molecular modeling, site-directed mutagenesis, and the attachment of dye molecules to a particular RC site. Correlated developments include refinement and simplification of the process for preparing reaction centers, studies of materials issues, atudies of device concepts, and transfer of RC preparation techniques to Biological Components Corporation.

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Photosynthetic Reaction Centers
as
Active Molecular Electronic
Components

Phase I Report SBIR A 92-103

August 19, 1993

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With Thanks to Professor Steven G. Boxer
Stanford University

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Photosynthetic Reaction Centers as Active Molecular Electronic Components

Introduction

Biological assemblies often bring together a large number of chemical constituents to produce unique functions. At the size scale defined by large protein molecules, such assemblies can be regarded as basic electronic, optical or mechanical components. Two examples of such assemblies, photosynthetic reaction centers (RCs) and bacteriorhodopsin are especially remarkable. Both systems are used by nature to pump charged particles (electrons and protons respectively) across non-conducting biological membranes in response to stimulation by light energy. The possibility that these molecular systems might be adapted for non-biological applications has been discussed widely in research publications, but up to the present all of the research effort aimed at finding and developing suitable applications has concentrated on bacteriorhodopsin as the active molecule. Because an increasing number of successful demonstrations of optical and electronic applications of bacteriorhodopsin are being reported in the literature, it is appropriate to use the experience and knowledge gained in these efforts to guide similar projects aimed at the application of photosynthetic reaction centers. BCC, the first American company set up to develop commercial applications of bacteriorhodopsin in holography, optical memory, light modulation and light sensing, has also begun efforts aimed at applying photosynthetic reaction centers. This report covers efforts in an initial study of possible applications of RCs.

Purpose of Project

Reaction centers (RCs) exhibit a remarkably high efficiency of extremely rapid, long-distance photoinduced charge separation under a wide range of conditions. The reaction center has electronic properties which are far superior, more flexible, and much less expensive to produce than any known synthetic donor/acceptor system. The latter are often discussed as prime candidates for components in molecular-scale electronic devices, but no actual applications or realistic assessment of their potential has been presented.

If molecular scale electronics is to become a significant new technology, it is necessary to develop approaches to overcome the difficult problem of connecting the active component(s) to a wire or to each other. The focus of the work in this first phase has been to develop a strategy for connecting photosynthetic reaction centers to electrode surfaces. This is a first, but essential, step in demonstrating the potential for fabricating light-driven molecular switches and novel sensors .

Background on Photosynthetic Reaction Centers

The RC is the smallest protein/chromophore complex which sustains the light-driven charge separation reactions initiating photosynthesis. It is a solid-state photochemical reactor consisting of an organized array of discrete components. Several years ago Michel crystallized the RC and with Deisenhofer and Huber solved the three dimensional structure to atomic resolution (Deisenhofer, et al., 1985). To date this is the only

membrane protein whose structure is known; these investigators received the 1988 Nobel Prize for their breakthrough.

The best characterized RCs are isolated from photosynthetic bacteria. The three dimensional structures of RCs from several species of bacteria have been determined to atomic resolution (Deisenhofer, et al., 1985; Boxer, et al., 1989; Boxer, 1990; Gunner, et al., 1986) this structural information provides the basis for our current understanding of the initial steps in photosynthesis. Bacteriochlorophylls and quinones are the prosthetic groups responsible for rapid electron transfer; the structure at various levels of detail is shown in Figures 1 through 3 along with further details on the components.

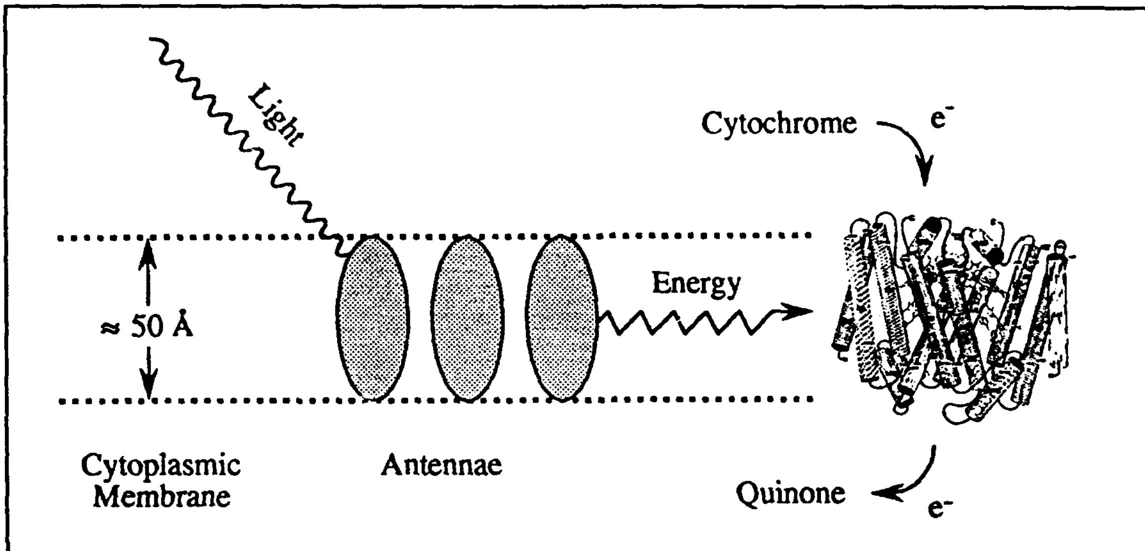

Figure 1. Schematic illustration of the architecture and dynamics of a native photosynthetic membrane. The antenna are chromophore-protein complexes which absorb light and transfer it to the reaction center on the right. Although the antennae enhance the absorption cross-section in natural systems, these proteins can be separated completely from the RC, and the RC will serve as its own antenna.

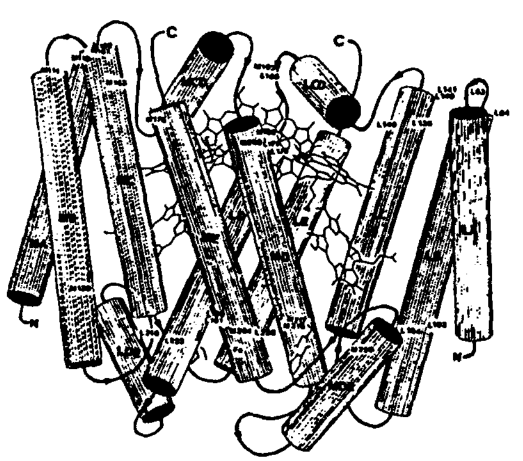

Figure 2. The reaction center protein as determined by x-ray structure analysis. The reactive components (see Figure 3 are encapsulated in two polypeptides, each consisting of ten membrane-spanning helices. A third polypeptide has one transmembrane helix and a large globular domain on the bottom side of the structure. This protein does not contact the reactive components directly and is not shown.

The precise steps are most easily understood by reference to Figure 3 which is a schematic representation of the redox-active components. The primary electron donor, P, is a pair of strongly interacting bacteriochlorophylls (BChls). It is often called the special pair, and its properties are those of the supermolecular dimer, not monomer. Following photoexcitation, ^1P transfers an electron within a few picoseconds at room or cryogenic temperatures to H_L, a monomeric bacteriopheophytin molecule (BPheo: BChl where the central Mg ion is replaced by 2H). The precise mechanism of this reaction, especially the function of the monomeric BChl labeled B_L, is the subject of current debate. H_L^- transfers an electron to Q_A within a few hundred picoseconds. Q_A transfers its electron to Q_B on the hundreds of picoseconds timescale. By this process the state $P^+Q_B^-$ is formed with essentially unit quantum yield, and it has a lifetime of several seconds. If Q_B is removed, the $P^+Q_A^-$ charge-separated state lives for about 100 ms. If Q_A is removed, the $P^+H_L^-$ charge-separated state lives for about 10 ns. Thus, the rate of formation of each intermediate is always several orders of magnitude faster than its decay ensuring a high quantum yield for each step.

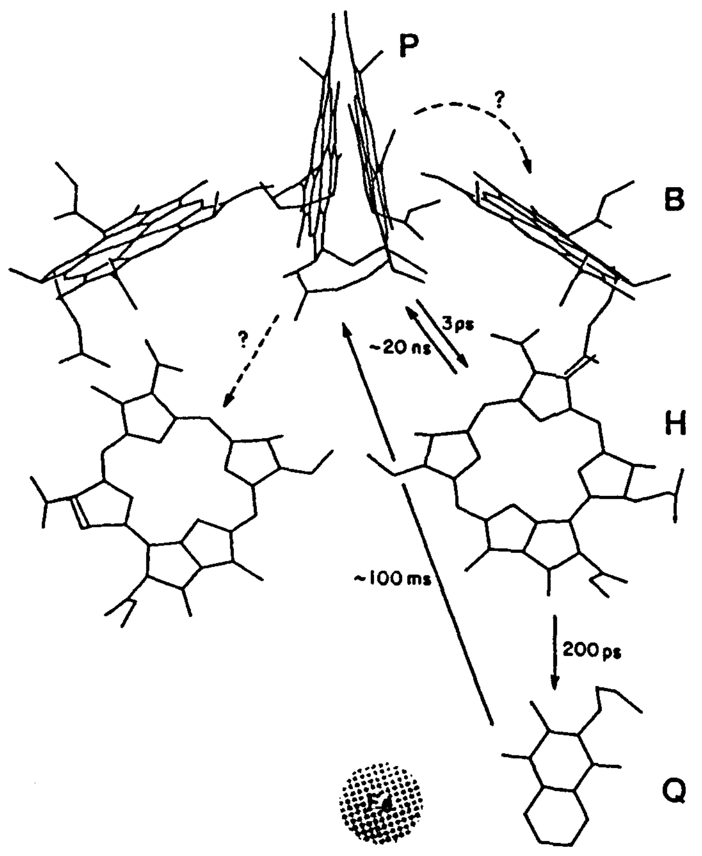

Figure 3. Arrangement of the redox-active components of the reaction center, as given by x-ray coordinates. These consist of the primary electron donor labeled P, intermediate electron carriers labeled B and H and the quinone electron acceptor, labeled Q_A. The approximate timescales of several reactions are indicated.

In its natural setting, the RC is embedded in a membrane bilayer. The hole on P^+ created by the process described above is refilled by electron transfer from a reduced cytochrome which is either bound to the RC or diffuses to it. Because the electron is on Q_B^-, the components on the right hand side as illustrated in Figure 3 are available to repeat the cycle described in the previous paragraph. This delivers a second electron to Q_B,

producing Q_B^-, which then picks up two protons, and leaves the RC complex as the neutral hydroquinone. This complicated process is essential for the biological function of the RC.

Aside from Q_B, two other chromophores are seen on the left hand side of the structure, B_M and H_M, which are BChl and BPheo, respectively. These components, along with the entire RC protein at the level of its secondary structure, are related to the components on the right hand side of the Figure by a local C_2 axis of symmetry running vertically from the center of P to the non-heme Fe atom. For reasons which are not understood, the initial electron transfer steps only occur along the right-hand (L- or sometimes A-) branch of redox-active components. Therefore in the following the subscripts on components will be dropped. This unidirectionality of electron transfer is an excellent example of exquisite molecular-level control of electron movement though an insulating medium. It is therefore a prime example of an elementary component in a molecular electronic device.

From the perspective of possible technological applications, several other aspects need to be highlighted. First, the reaction forming P^+ from ^1P leads to the complete loss of the strongly allowed, lowest energy electronic transition, known as the Q_Y transition, which occurs at approximately 900 nm. Thus, whenever the RC is in the P^+ state, the near infrared absorption spectrum is very different from the neutral state. The changes in the near infrared region are illustrated in Figure 4.

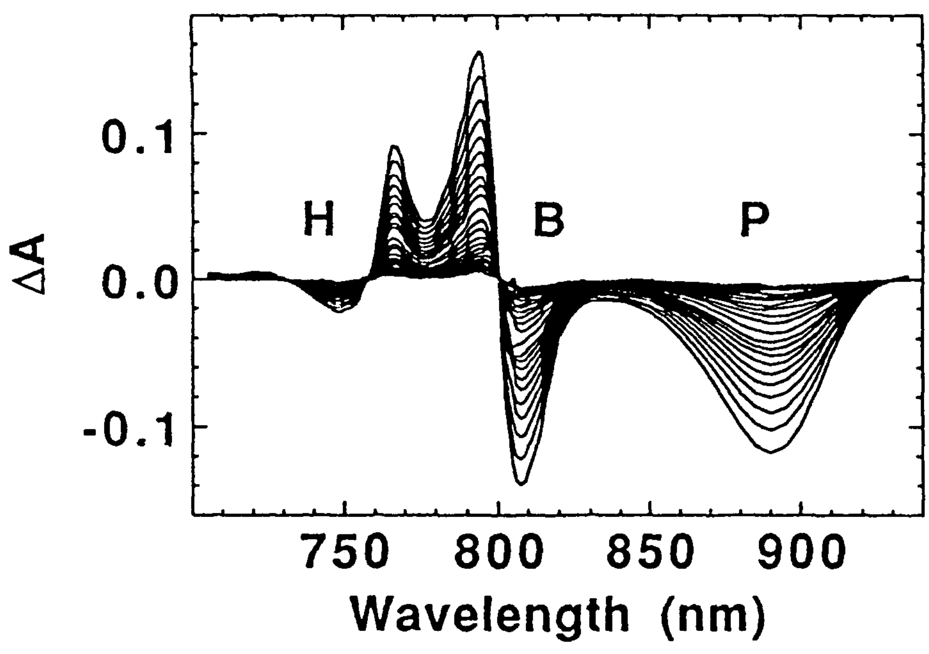

Figure 4. Transient absorption spectrum on the millisecond time scale, illustrating the changes that occur in the near infra-red region following excitation of reaction centers. The bands are labeled according to the chromophores shown in Figure 3. A large net change in absorption (bleaching) is observed for the special pair P. Bandshifts are observed for the B and H bands due to electrostatic interaction with the P^+Q_A dipole.

In addition, P^+ has a characteristic absorption at about 1250 nm which is absent in the neutral state. The oscillator strength of this absorption is much smaller (10-50 times) than that of the Q_Y transition of P. A second key feature is that all of the components in Figure 3 are encased in two polypeptides. These polypeptides bind the redox active components tightly, protect them from exogenous impurities, fix their positions so that diffusion cannot occur, and provide some level of control over the redox properties of the chromophores (see below). A third polypeptide known as the H-subunit is also associated with the RC complex; however, none of the redox-active components interacts with this protein. The genes which code for the L- and M-polypeptides have been cloned and placed on plasmids which allow for efficient production of RCs in strains where the native RC genes are deleted (Coleman and Youvan, 1990). Thus, it is possible to change individual amino acids which interact with the redox-active components or larger segments of the protein sequences using standard recombinant DNA methods.

Technical Relevance of the Project

This study addresses several of the key conditions which must be met before a material such as photosynthetic reaction centers can be used in photonic or sensor devices. The first condition is that RCs have the appropriate optical and electronic properties for the intended application. The second condition is that the reaction center be deposited on a metal or semiconductor surface in such a manner that directed electron transfer will occur during the photocycle. The third condition is that the deposited RCs will not degrade to an appreciable degree under the operation of the device under normal conditions.

Electronic and Optical Properties of Reaction Centers

First, the charge separation and recombination reactions are essentially activationless. In fact the rates of all reactions increase slightly as the temperature is lowered, with no loss of quantum efficiency. This is in sharp contrast to synthetic donor/acceptor systems which have been prepared for molecular electronic applications. Such systems are not only extremely difficult and costly to prepare, but they are generally unstable and in nearly all cases cease charge separation when placed in a solid matrix (essential for many technological applications) or cooled.

Second, the lowest electronic excitation occurs around 850-1000 nm (see Figure 5), depending on species. This wavelength range is very well matched to existing laser diodes, and this is one of the few molecular systems capable of highly efficient, reversible photochemistry using such low energy photons. Since there are several chromophores in the RC, appreciable absorption occurs throughout the visible and near-ultraviolet regions. Energy transfer among the chromophores to the primary electron donor (P in Figure 3) is even faster (tens of femtoseconds) than electron transfer, so excitation at all absorbed wavelengths leads to excitation of P and to the same highly efficient charge separation.

Third, the charge separation and recombination reactions can be manipulated with external electric fields (Boxer, et al., 1989; Frazen, 1991), making it possible to control the lifetime of intermediates. Because the absorption spectrum of the P^+ state is very different from the neutral ground state (see Figure 4), it is also possible to manipulate the optical properties of the RC with external electric fields.

Fourth, the native quinones can be replaced with many different quinones thereby altering the rates of charge recombination reactions. Also, non-quinoid, aromatic molecules can

be substituted into the quinone binding sites. If both quinones are present, the charge recombination time is extended to one second.

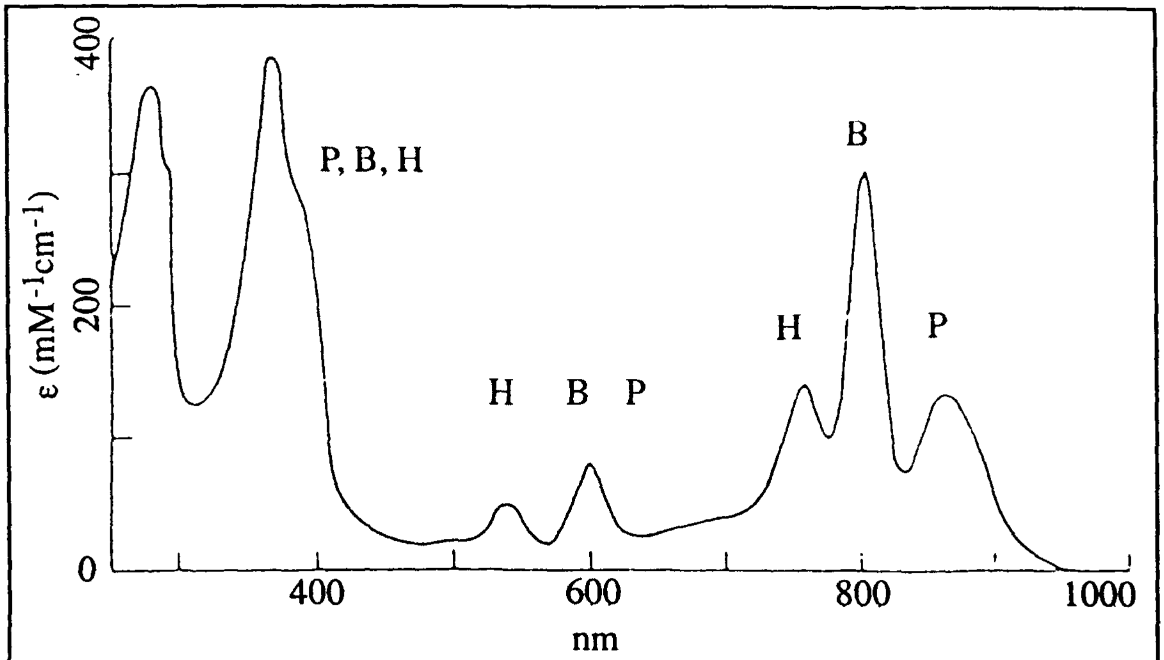

Figure 5. Absorption spectra of reaction centers from Rb. sphaeroides or Rb. capsulatus. Absorption of light at any wavelength shorter than about 900 nm leads to efficient charge separation forming P^+. This leads to a bleaching of the P absorption band which recovers when charges recombine (c. f. Figure 4).

Thin Film Technology

With respect to the compatibility of RCs with thin film and deposition technologies, we note that the RC complex can be suspended in a wide range of media without degraded function. For example, RCs embedded in thin plastic films are very stable and fully functional. Furthermore, the genes for the two principal RC proteins (see Figure 2) have been cloned, strains of bacteria in which the native RC genes are deleted have been constructed, and highly efficient expression systems have been developed (Coleman and Youvan, 1990). Therefore the protein primary sequence can be manipulated by recombinant DNA methods, so the molecular scaffolding can be altered. It is therefore possible to produce modified RCs as easily as it is to produce the native RC. Specific modifications will improve orientability in an electric field or form sites for attachment to particular substrates.

Stability of Reaction Centers

As far as stability is concerned, the electron transfer reactions indicated take place within the chromophore/protein complex, thus the protein is not only the solvent for these reactions but also provides a molecular scaffold. As a result, the reactive components and intermediates are protected from undesirable side reactions, and the charge separation and recombination steps are highly reversible and can be repeated indefinitely. Furthermore, thin film technologies are available which can be applied to the problem of stabilizing reaction centers.

Project Objectives

The goal of this Phase I SBIR has been to develop strategies for the covalent attachment of reaction centers to electrode surfaces and to other reactive components. The key to our approach has been to use the known three dimensional structure of the reaction center and molecular modeling to decide among the many possible approaches, by evaluating the limitations and opportunities of each approach.

The specific objectives of this Phase I proposal are to:

a. Identify sites on the surface of reaction centers in order to covalently connect the reaction center to electrode surfaces or other reaction centers. The design principles require that the attachment sites be near to P and Q and compatible with the reaction center topology. This protein engineering will be done largely by computer graphics modeling.

b. Develop specific concepts and designs for molecular devices in light of the protein engineering opportunities and limitations developed above. This includes light-driven molecular switches and biosensors.

c. Initiate the transfer of reaction center production technology and molecular biology from Professor Boxer's laboratory at Stanford University to BCC.

Work Performed

We identified six technical tasks In our Phase I proposal.

Task I. Identification of sites for attachment based on molecular modeling and electron transfer theory.

Task II. Optimization of reaction center attachment strategies.

Task III. Reaction center stabilization strategies.

Task IV. Development of concepts and designs for light-driven switches and biosensors.

Task V. Preparation of reaction centers.

Task VI. Initiation of site-specific mutagenesis based on strategies developed in Tasks I-IV.

We have changed the order of discussion in the subsequent report as follows: Task I is discussed under the title 'Identification of Sites for Attachment. Tasks II and VI are discussed below under 'Strategies for Attachment of Reaction Centers. Task IV is discussed in the section on 'Device Concepts'. Task V is discussed in the section entitled 'Preparation of Reaction Centers. Task III is discussed in the section on 'Stabilization of Reaction Centers. We have also documented our current knowledge on preparation of reaction centers and site directed mutagenisis in Appendices A and B.

Identification of Sites for Attachment

Task Description.

For the purpose of discussion, RC structure can be crudely represented as a cylinder (Figure 6.). The upper and lower discs correspond to the aqueous interfaces. Electrons are delivered and removed from the RC at these interfaces during its natural function, and these are the logical sites for attachment to external components.

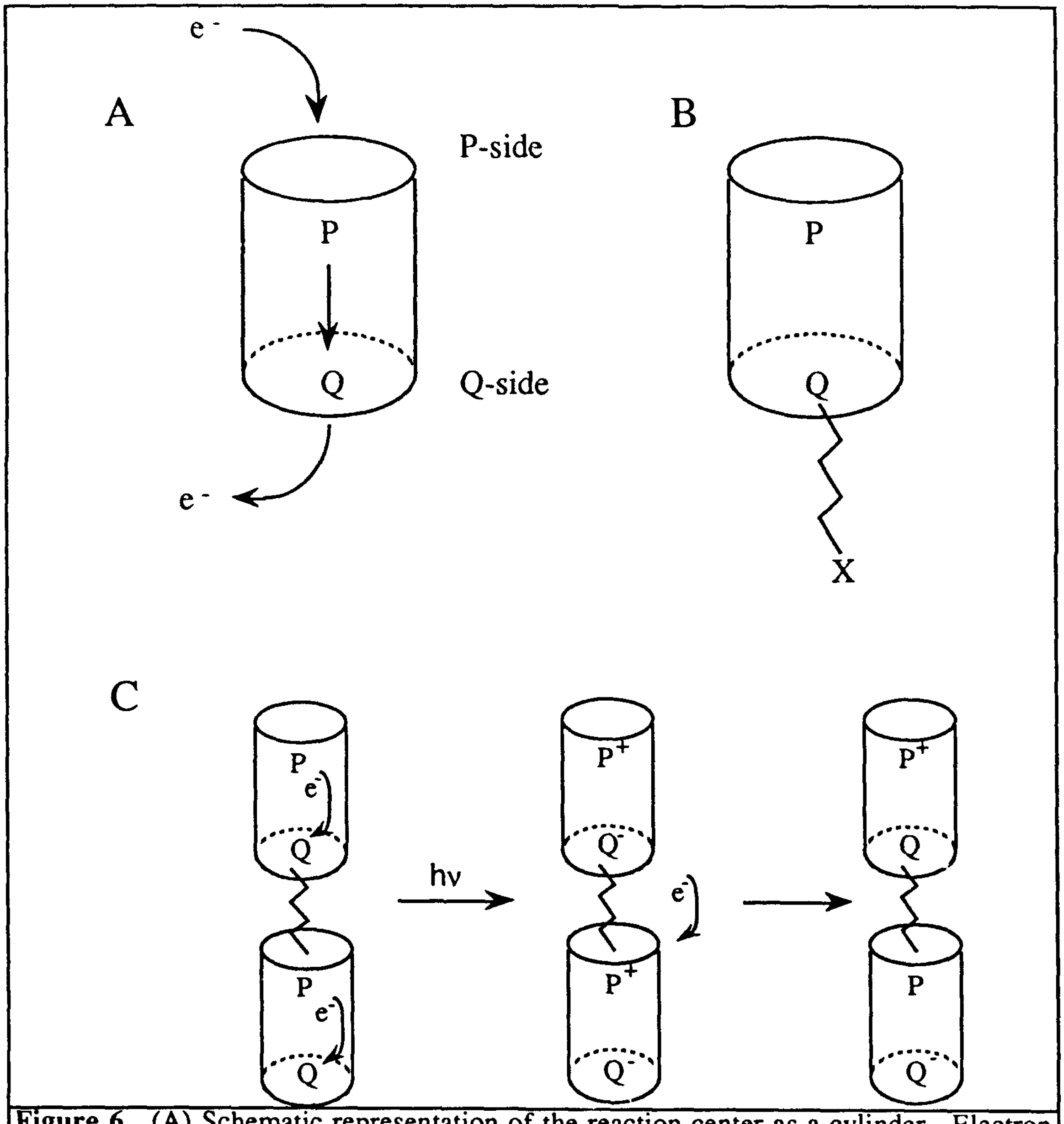

Figure 6. (A) Schematic representation of the reaction center as a cylinder. Electron transfer is from P to Q. (B) Introduction of a Q-side attachment site X by reconstitution with a functionalized quinone. (C) Covalent connection of RCs in series. Electron transfer between RCs following absorption of two photons is illustrated.

In the geometry illustrated in Figure 6, the upper aqueous surface is closest to the primary electron donor, P, and will be referred to as the P-side. The lower surface is closest to the quinone electron acceptors and will be referred to as the Q-side.

There are many possible sites on both surfaces for engineering unique connections. Because the purpose of the connection is to make possible direct or facilitated electron transfer either to P^+ and/or from Q^- following light-induced charge separation, design principles are required. The first of these is the distance dependence of electron transfer rates. The rate of electron transfer falls off roughly by one order of magnitude for each 1-1.5 angstrom of increased distance between donor and acceptor. This places severe constraints on sites of connection, which should therefore be at or near the surface residue which is closest to the redox-active target. On the other hand, there are constraints on the protein surface related to surface topology. For example on the P-side of the RC, the region directly above P is where cytochrome binds during native function, so there is a depression in the surface. Insertion of a unique binding residue at the bottom of this depression may lead to an attachment site which is not accessible above the plane of the P-side surface. Likewise on the Q-side, the H protein subunit, which is more difficult to modify by site-directed mutagenesis, may be the site closest to the quinones.

Because chemically unique and reactive amino acids such as cysteine can be inserted at any position in the primary sequence by site-directed mutagenesis, our first task was to identify and optimize possible sites for initial modification. We accomplished this by using the atomic coordinates of the RC and molecular modeling.

Molecular Modeling.

Several potential sites were identified, based on the following criteria: (i) Attachment site should be unique in order that later processing steps produce a unique orientation of the RC. (ii) For compatibility with recombinant DNA techniques, attachment should be by means of a thiol group, i. e. by substituting a cysteine residue. (iii) The site should be close to the P-surface and exposed to the aqueous surface of one of the proteins. (iv) The residue selected for substitution should be similar to cysteine, in order that substitution of a residue does not affect the protein folding.

Examination of the x-ray structure by computer graphics suggests that (M)189 is surface-accessible and also close to P. The surface region of the RC near P has a depression or crater which likely serves as the concave surface for binding to the convex surface of cytochrome, which is a small globular (roughly spherical) protein. Residue (M)189 is on the edge of this crater. This may prove to be important for attachment of the RC to relatively flat surfaces. Finally it has often been found that modification of M-polypeptide residues is less disruptive than modification of L-side residues. Figures 7A and 7B are illustrative of the computer modeling studies performed in Steve Boxer's laboratory. The atomic coordinated for these modeling studies have been transferred to BCC.

A.

B

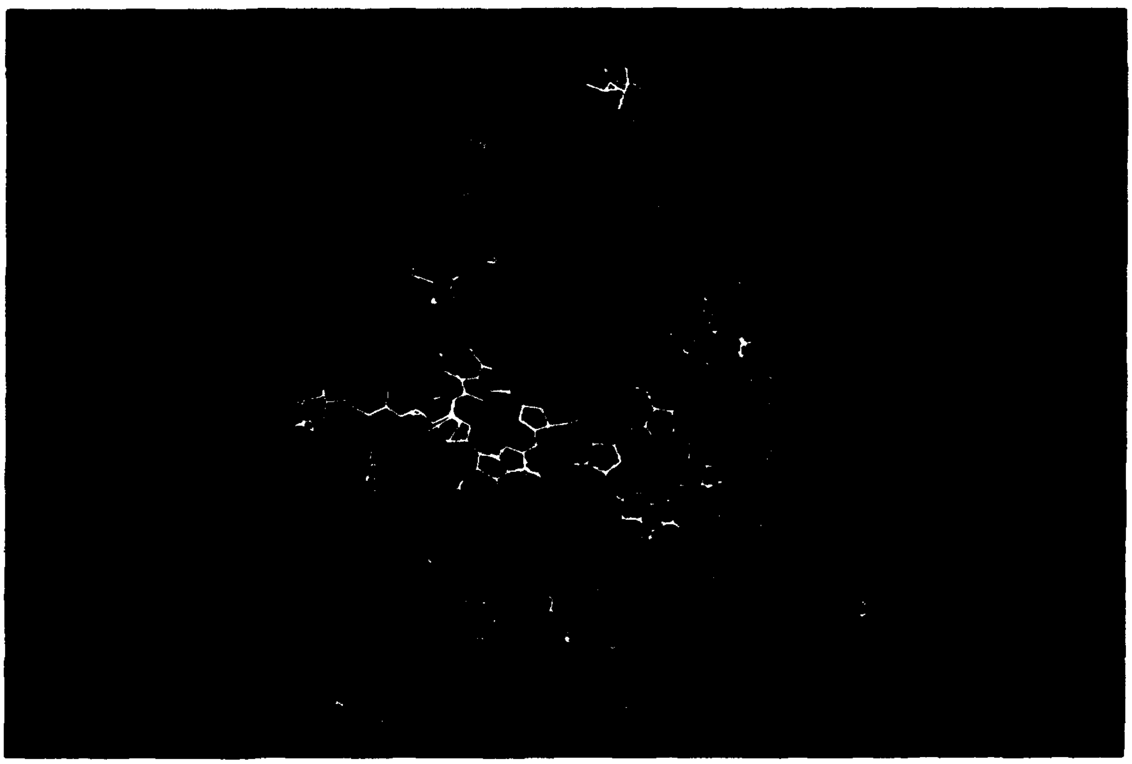

Figure 7. Computer graphics showing reaction center chromophores and the location of site of attachment near P, (M)189, near red spheres. A. Active components and the M-protein. B. Active components alone.

Strategies for Attachment of Reaction Centers

Because the RC is potentially a versatile component in a variety of settings, we have considered covalent connection to a number of smaller redox-active components, as well as to electrode surfaces. These can be broadly classified as i) coupling to small-molecule redox reagents on the P- or Q-side surfaces, ii) coupling to small-molecule redox reagents on the P- or Q-side surfaces and iii) coupling between RC surfaces.

COUPLING TO SMALL-MOLECULE REDOX REAGENTS ON THE P- OR Q-SIDE SURFACES. There have been many investigations during the past few years describing relatively small redox-active molecules which can be connected simply to proteins. An intensive search of the literature describing these molecules was undertaken to discover candidates whose redox potentials are matched to those of the RC. The most versatile appear to be transition metal sepulchrates as their redox potential can be varied at will and they offer the possibility of serving as a bridge between the RC and the surface.

COUPLING TO SMALL-MOLECULE REDOX REAGENTS ON THE Q-SIDE USING POLYQUINONES. The Q-side offers an alternative opportunity for making a direct electronic linkage. It is straightforward to remove the quinones and then replace Q_A with many other quinones or related aromatic molecules. Therefore it is possible to introduce a redox-active linkage to the Q-side of the RC by attaching the linker to the quinone which is reconstituted into the Q_A binding site. Schematically this is illustrated in Figure 6B. This principle can also be readily extended as an approach for preparing a direct Q-side linkage to an electrode surface.

COUPLING BETWEEN RC SURFACES. A potentially significant application of the technology described here is to connect RCs in series in a head-to-tail fashion. This is illustrated schematically in Figure 6C. As illustrated, it should be possible to transfer electrons between RCs generating giant dipoles in response to sequential excitation of both members of the RC dimer. This would be a highly non-linear electrical response to photons with the potential for moving electrons over very large distances. In order to achieve this, elements from the strategies for attachment at the P- and Q-sides need to be simultaneously optimized with special attention to the interfacial region between the two proteins. This can be achieved by careful computer graphics modeling. As discussed below RC dimerization, though in the incorrect orientation, was accidentally discovered in the course of funcionalization.

Technical Issues

Many applications depend on immobilization, targeting, and redox contact with the RC. The distance from P to the quinone Q is 25Å; the aqueous surfaces on both sides are 7Å above and below these components. The best sites of attachment have been evaluated in Tasks I and II. The eventual objective is to covalently connect a modified RC to a gold electrode. This concept is illustrated schematically in Figure 8 and is discussed below. In order that we can make a useful device from reaction centers bound to a conductor such as a metal layer or a wire, several competing effects must be balanced. First, because the RC is a membrane protein, RCs must be solubilized in a detergent. The detergent may interfere with the process of bringing reactive sites on the aqueous surface of the protein close to reactive sites on the substrate. Therefore, the substrate reactive sites must be placed on molecular chains extending from the substrate surface. If the chains are too long, tunneling rates from a conductive substrate to the RC will be reduced. This should not be serious for conjugated chain lengths less than 20Å, but we need to verify this experimentally.

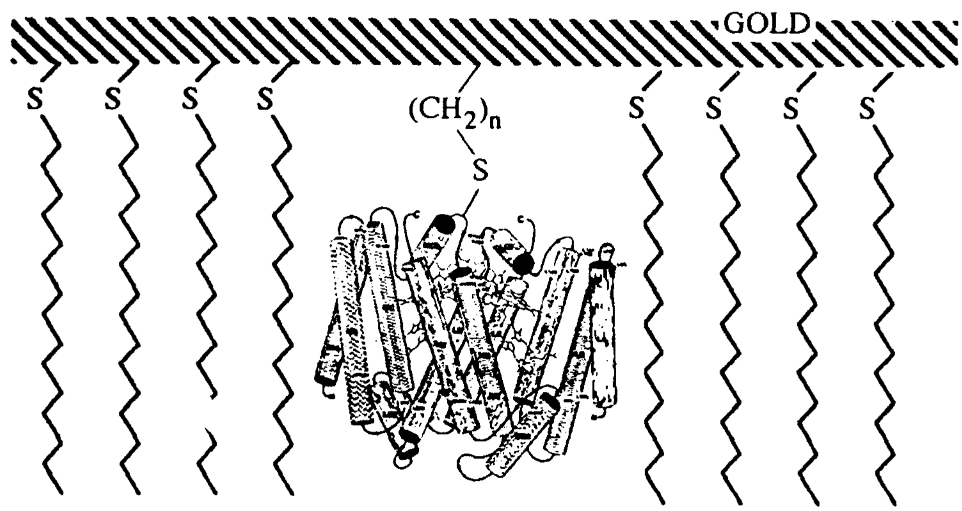

Figure 8. Schematic illustration of covalent specific attachment of reaction centers to electrode surfaces. Following photo-induced charge separation within the RC electrons flow from the electrode into P^+.

Substrates

During the past two or three years several groups have perfected the modification of the gold 100 surface using thiols. Thin films of gold, consisting largely of extended regular regions are easily prepared by metal vapor deposition, a technique we use routinely. We have introduced a unique cysteine residue into the sequence of one of the RC polypeptides (see below), engineered to be as close to P as possible, to serve as a point of attachment to a well-defined electrode surface. It is unlikely that direct attachment will succeed as this would bring the RC complex too close to the metal surface; however, a simple conjugated chain as illustrated, can be used to extend the distance as desired or until a stable, linked complex is formed. The remainder of the surface would be covered with simple alkyl thiols, which should stabilize the RC complex as in a membrane (RCs can be solubilized in hexane). Because of the proposed approach to fabrication, the relevant kinetics and stability at each step can be evaluated quantitatively to optimize the rates.

One alternative is to use porous films as a means for overcoming the difficulties of attaching large molecular groups to a flat surface. The basic idea is to deposit organic materials into the pores of a specially fabricated porous alumina layer. There are several reasons for attempting to do this, among which are: i) pores in the film could be tailored to the size of the molecular assembly, ii) simple techniques exist for the deposition of organic molecules in the pores, iii) molecules trapped in the entrance to a pore would be nicely positioned for investigation by scanning probe microscopy, iv) the aluminum oxide matrix is rigid, so the molecules would be well localized, v) we should be able to isolate a single molecule in a pore so we can measure single electron transfer (see below in the section on device concepts) vi) the aluminum oxide matrix should afford some measure of protection of the organic materials.

Microporous membranes can serve as templates for the fabrication of nanostructures. Martin (1989, 1991) reports on the synthesis of polypyrrole microtubules in

commercially available Nuclepore polycarbonate filters. Brumlik and Martin (1990) have also described the use of conventional silanization chemistry to attach specific molecular anchors to the pore walls of nanopore alumina membranes. This step is followed by the synthesis of specific polymers which can bind to these anchors, within the pores of the membrane. Microporous membranes can serve as templates for the deposition of metals. Metal-insulator composites of varying metal fraction can be prepared by electrochemical deposition of gold onto porous aluminum oxide membranes Foss, et al. (1992).

The basic idea for the deposition of photosynthetic reaction centers in microporous membranes is relatively simple. These membranes can be prepared by the deposition of gold on one side of the membrane and along the walls of the pores, as in Figure 9. We can then deposit large molecules or molecular assemblies such as reaction centers into the pores of the composite membrane, by using the membrane as a filter and driving a fluid dispersion of the molecules through the pores. It may also be possible to prepare the deposited gold (see the discussion in the substrates section) with chemical groups which will bind with the reactive groups placed on the protein. After deposition of the molecules, a scanning tunneling microscope can be used to image the RCs trapped in the entrances to the pores.

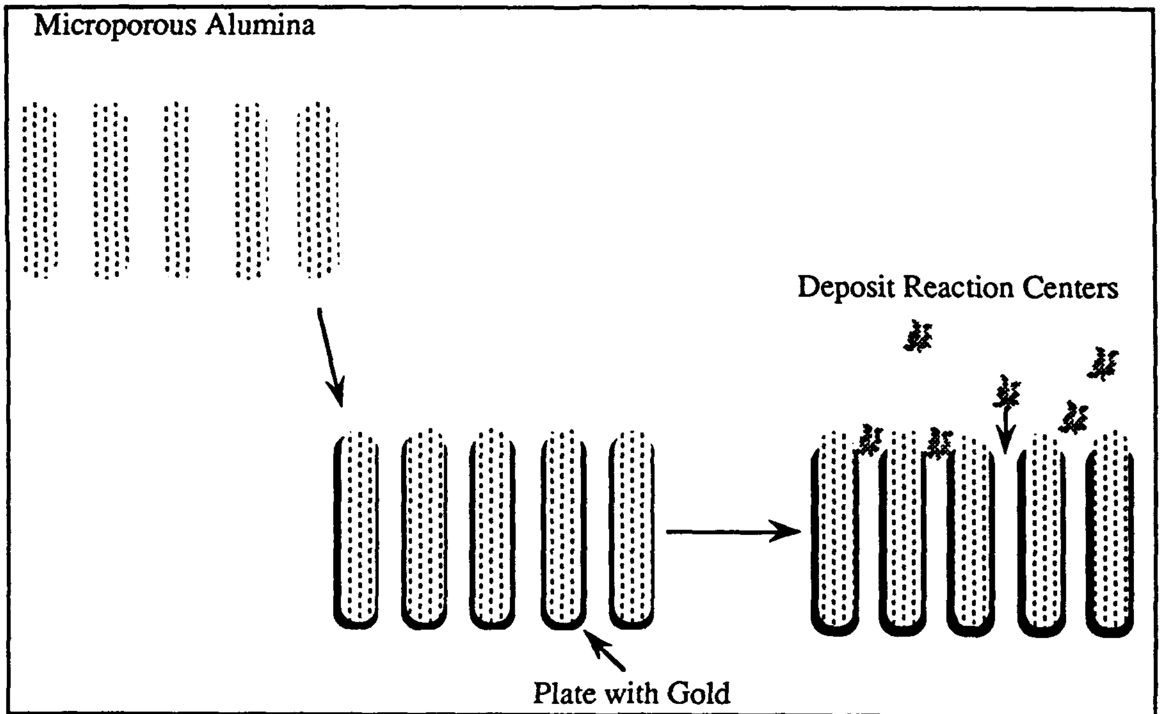

Figure 9. Process steps in fabricating composite microporous film and trapping reaction centers in its pores.

Initial Experiments

The following section describes experiments performed in Steve Boxer's laboratory. Rb. capsulatus reaction centers were prepared from the strain in which the Leucine residue at position M189 was replaced with a cysteine by site directed mutagenesis. This is the only cysteine residue on the aqueous surface of the protein. It is located directly above the special pair, and was chosen as the most exposed surface site which is closest to the

special pair. Because of the presence of the free sulfhydryl group on the protein surface, reaction center dimers form quite readily in the presence of air and traces of metals in solution. These dimers are not interesting in themselves as they represent "head-to-head" dimers, rather than "head-to-tail" dimers; nonetheless, their existence proves that close, covalent connection of RCs is possible, and this may ultimately be exploited for the fabrication of "head-to-tail" arrays for the production of very long distance charge-separation, as outlined schematically in Figure 6C.

Initial attempts to attach dye molecules were entirely unsuccessful. By consultation with colleagues in the biotech industry, it was discovered that bacteria often modify free sulfhydryl residues. This modification can be easily reversed by treatment with reducing agents, and the same reducing agents are also required to separate any dimers which have formed. It was also discovered that addition of EDTA, which complexes metal ions, greatly reduces subsequent disulfide formation. All of these procedures are simple to perform and are part of our standard work-up procedure.

Under these conditions, attachment of dye molecules which uniquely label sulfhydryl groups proved to be simple and quantitative. The dye 5-IAF (5-iodo-acetamide fluorescein) from Molecular Probes was used. This dye has a strong absorbance around 498 nm, in a region where the RC absorption is relatively low. The dye and RC were mixed together and incubated for one hour. The mixture was then loaded on an ion-exchange column at low ionic strength, where the unmodified and modified RCs bound, but the dye passes through the column. After elution of the excess dye, the RC fraction was removed with an ionic strength gradient. It was found that wild-type RCs eluted identically before and after treatment with the dye, and there was no evidence for bound dye at 498 nm. By contrast, the mutant eluted much more slowly from the column after treatment with dye than before, and the absorption spectrum showed a clear peak at 498nm whose wavelength and amplitude corresponded to quantitative addition of 1 equivalent of dye. The change in elution from the column is the result of the addition of a negative charge associated with the dye on the protein surface. This provides a useful strategy both for purifying the RCs and for orienting them in an electric field.

In addition to the fluorescein dye, iodoacetamide conjugated with malachite green was attached to RCs. This dye has the opposite charge, and, as expected, it eluted rapidly from the ion exchange column at low ionic strength. As with the fluorescein dye, it was demonstrated that 1 equivalent bound to the RC surface. These experiments demonstrate that small molecules can be quantitatively attached to the complex protein surface, and that dyes of opposite charges can be attached. In both cases, it was shown that the internal function of the RC, as assessed by formation of the P^+Q^- charge-separated state, was normal and that the absorption of all RC chromophores in the near infrared was unaffected by surface attachment. Thus, position 189 is an attractive target for attachment to electrode surfaces and this is in progress.

Device Concepts

Reaction centers should really be thought of as a self-contained molecular device capable of very rapid, light-initiated electron transfer. Broadly speaking, the opportunities to be addressed involve exploiting these features in the fabrication of more complex devices. In the following we summarize briefly several of the directions considered during the grant period. The discussion of single electron devices in this section follows Devoret, et al, 1992.

Single Electron Devices

Electrons can be made to pass through a circuit one by one in nanoscale devices. The basic components of such devices are tunnel junctions and capacitors. We will discuss the properties of progressively more complex circuits: first, a tunnel junction, then an electrode isolated from a voltage source by a tunnel junction and a capacitor, and finally a single electron transistor.

TUNNEL JUNCTIONS. A tunnel junction is two metal electrodes separated by an insulating layer thin enough that electrons can tunnel across. Tunneling can be regarded as an all-or-nothing process because electrons spend a negligible time under the potential barrier corresponding to the insulating layer. Without some additional circuit elements, this all or nothing behavior is not apparent. For example, if one applies a voltage V to a simple tunnel junction, electrons will tunnel randomly across the barrier at an average rate equal to V/eR_t, where the tunnel resistance R_t depends upon the area and thickness of the insulating barrier. Although the transport of electrons through the tunnel junction and through an ordinary resistance are fundamentally different, the current-voltage characteristics are both linear.

ISOLATED ELECTRODES. If, instead of applying a voltage source directly to the junction, one biases it with a voltage source U in series with a capacitor C_s, an entirely different behavior emerges. A metallic electrode entirely surrounded by insulating material is formed between the junction and the capacitor (see Figure 10). This "island" electrode is coupled with the rest of the circuit by the capacitance of the junction, C, and the capacitance C_s. Under an applied voltage, the two capacitances will have charges Q and Q_s respectively. Because charge can leave the isolated electrode only by tunneling, the instantaneous total charge on the island, $Q - Q_s = ne$, becomes an integral multiple of the charge of a single electron. If the island dimensions are small enough so that the electrostatic energy $E_c = e^2/2C_\Sigma \equiv e^2/2(C + C_s)$ is larger than the characteristic energy, k_BT, of thermal fluctuations, then in principle the measured charge on the island varies in discrete steps.

When U = 0, the number of electrons, n, on the island stays identically zero, because the entrance or exit of an electron would raise the electrostatic energy of the island to a level higher than permitted by thermal fluctuations. As U increases from zero, the total energy difference between the n = 0 and n = 1 states of the circuit decreases, because when an electron tunnels to the island, the potential drop C_sU/C_Σ partially compensates the electrostatic energy of the island. The total energy, E, of the circuit is given by $E = E_c(n - C_sU/e)^2$, therefore when $U = e/2C_s$, the n = 0 and n = 1 states have equal energy and an electron can tunnel in and out freely. As U is increased the n = 1 state becomes the lowest energy state. The maximum stability of the n = 1 state against fluctuations is reached when $U = e/C_s$. In this case the charge Q vanishes. Each time the voltage U is increased by e/C_s the number of excess electrons on the island is increased by one. A plot of $\bar{n}$, the average of n, gives a staircase as in Figure 10.

As the temperature is increased, the staircase becomes rounded, and for temperatures such that $k_BT >> E_c$, the graph approaches a straight line. Typical experimental conditions for metallic junctions are temperatures of 50 mK for a junction of 50 X 50 nm. The quenching of island charge fluctuations has been demonstrated experimentally (Lafarge, et al, 1981). Other experiments (discussed briefly below) show that these effects hold down to a few nanometers, and for these dimensions single electron transfer is observable at room temperature. For structures below a size of a few nanometers, one question (from the point of view of circuit engineering) is whether above discussion applies.

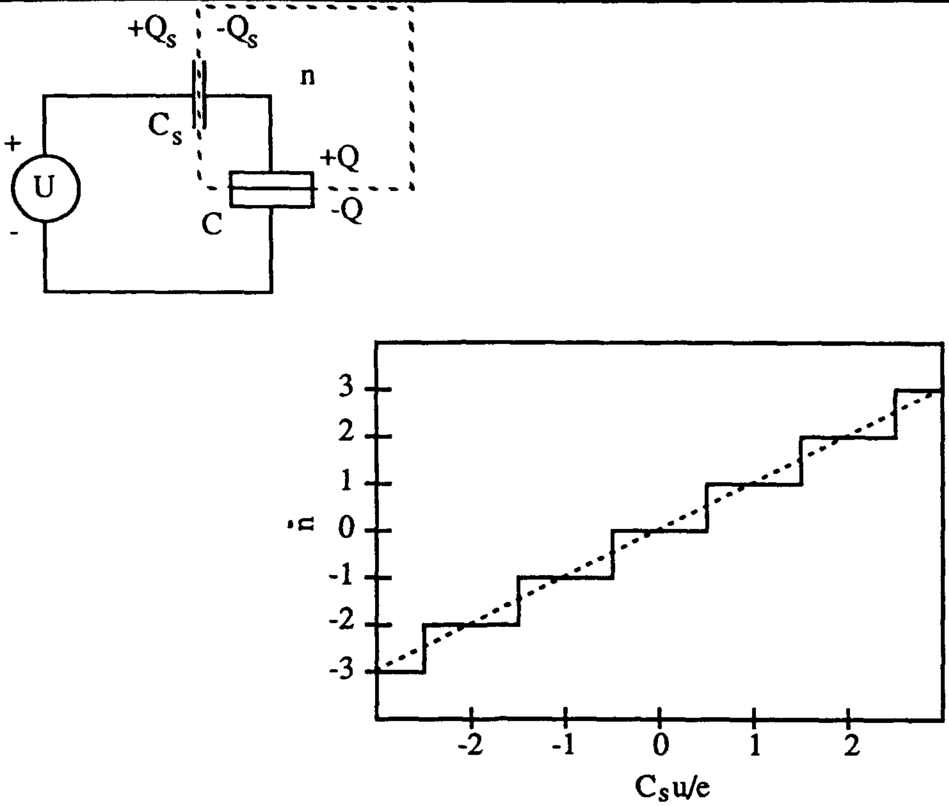

Figure 10. (From Devoret, et al, 1993)The circuit is a junction biassed by a voltage source U in series with a capacitance C_s. The metal electrode between the junction and the capacitance forms an isolated island (box in dashed line) which contains n excess electrons. The graph shows the variation of $\bar{n}$, the average of n, as a function of U when $k_BT \ll E_c$ (solid line) and when $k_BT \gg E_c$ (dashed line).

We know that similar effects may be observed in single molecules or small molecular assemblies; the question is whether it makes any sense to talk about capacitance, or at least factors such as the dielectric constant, which determine capacitance. If so, it would be easier to take advantage of some of the attractive features which emerge at molecular dimensions. For example, at the molecular scale capacitances (or effective capacitances as determined by effective dielectric constants) should become very small, and the electrostatic energy predicted by the equations above is greater the characteristic energies of thermal fluctuations at room temperature. The photosynthetic reaction center offers an example of the effect of dielectric constants on electron transfer. After the initial photoexcitation the electron always ends up on the same quinone. Experiments performed by Steve Boxer indicate that the effective dielectric constants seen by the two quinones are different, and thus there is a difference in energy between the two transfer pathways. This energy difference is enough to account for the preferred pathway. If effective dielectric constants could be controlled in analogous devices, this might be a way to direct electron transfer in nanoscale circuits.

SINGLE ELECTRON TRANSISTORS. The one junction one-island circuit is the simplest in which single electron transfer can occur. This circuit does not produce an externally measurable static current. We complete our discussion by describing a circuit which has an additional tunnel junction and voltage source. In this circuit, the isolated electrode has the same property described above of storing an integral number of electrons, but in

addition the presence or absence of extra electrons modulates the current flow. This more complex two-junction, one-island, is shown in Figure 11. The state of the circuit is characterized by the number of electrons N and N' which have passed through the two junctions. Following Devoret, et al, the number, n, of excess electrons on the island and the number, p, of electrons transferred from one terminal of the voltage source to the other are given by $n = N - N'$ and $p = (N + N')/2$ respectively. We denote the state of the circuit by the pair (n, p). Because the precise value of p is irrelevant in the most of the following discussion we will simplify the notation for (n, p) to (n) and the notation for (n, p+1) to (n)*.

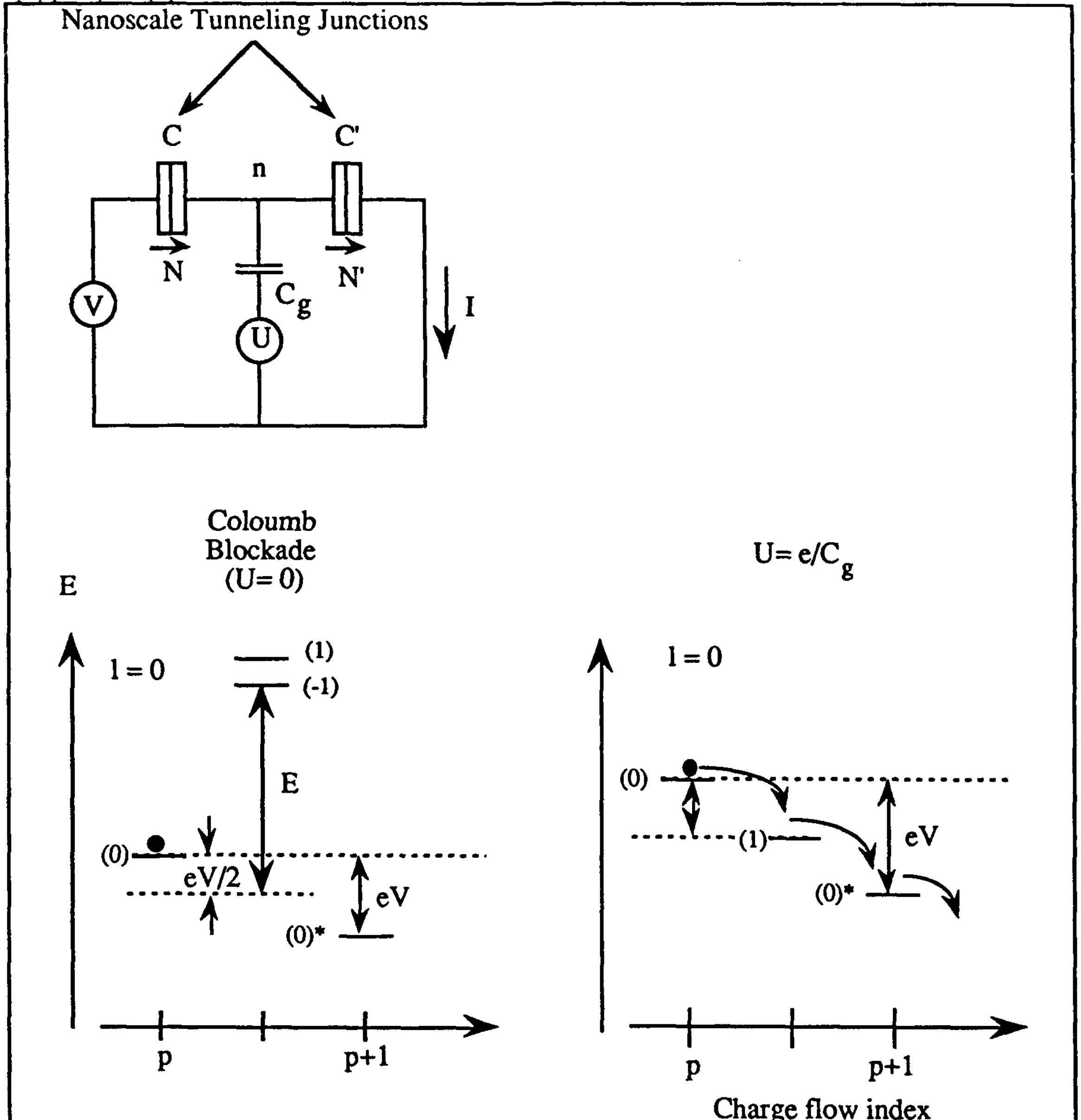

Figure 11. (From Devoret, et al., 1993) Schematic of single-electron transistor (SET), and energies of the states when $U = 0$ and $U = e/C_g$. The numbers in parenthesis are the values of the number n of excess electrons on the SET island. The charge flow index is half the sum of the numbers N and N' of electrons that have traversed the junctions. In the case e/C_g no current can flow through the device: this is the Coloumb blockade.

The energy of the state (n, p + 1), is lower than the energy of the state (n, p) by eV, so ordinarily one might expect electrons to flow through the circuit as long as a non-zero voltage is applied, so the circuit has no absolutely stable states. To go from state (n) to (n)*, however, the circuit must go through state (n+1) or (n-1) because tunnel events are discrete. States (n+1) and (n-1) differ from state (n) by an electron tunneling event through the first or the second junction. These events change the energy of the circuit. The relevant quantity is the single electron Coloumb energy, given by $E_c = e^2/2C_\Sigma \equiv e^2/2(C + C' + C_g)$. We assume that $eV << E_c$ in order to simplify the discussion.

When the control gate voltage, U, is set at U = 0, the energy of states (-1) and (1) will be $E_c - eV/2 \approx E_c$ above the energy of state (0), as in Figure 11. At low temperatures this provides a Coloumb barrier for transport of electrons around the circuit. This is the so-called Coloumb blockade. When the control voltage is given by $U \approx e/2C_g$, states (0) and (1) have nearly the same energy (see Figure 11). As soon as the energy of the (1) state is below the energy of the (0), a (0) -> (1) transition occurs, and an electron tunnels through the first junction. If U is set so that the energy of the (1) state is between the energies of the (0) and (0)* states, the transition (1) -> (0)* occurs and the electron tunnels through the second junction. This takes the circuit back to the initial electrostatic state and the cycle can repeat. This cycle produces a current on the order of $V/(R_t + R_t')$. If U is increased the circuit enters a new Coloumb blocked state with one excess electron on the island. The Coloumb blocked states are in one-to-one correspondence with the steps of Figure 11.

In practice, a current on the order of 10^9 electrons/second can be switched on and off by the presence of half the electron charge on the gate capacitor, hence the name 'single electron transistor' (SET) has been given this device. The charge sensitivity of the SET is six orders of magnitude better than conventional FET electrometers. Possible applications are the detection of individual photoinduced electron-hole pairs in semiconductors, or the transfer of electrons through a small patch of RC film. The latter device could form the basis of a family of extraordinarily sensitive photodetectors or chemical sensors. These devices could operate without cryogenics, as Coloumb blockade at room temperature has been reported in a device similar to that in Figure 11.

It should be noted that more complex devices have been also proposed and investigated. Esteve (1992) and Pothier et al (1992) describe such devices as single electron pumps and turnstiles. We believe that it would be profitable to consider replacement of one or more components of these circuits by RCs in order to produce photo-activated devices. We discuss some of the issues involved in the design of a chemical sensor using RCs below.

An RC wired as illustrated in Figure 12 can serve as a discrete molecular component in a more complex system. A first step is to demonstrate light-induced photoconductivity proceeding through the discrete set of molecular components contained in this array, with the hole on P^+ being replaced by an electron from the gold electrode. This would be a major technological achievement. Recent advances by several groups have demonstrated approaches to fabricating extremely fine (tens of nm) metal wires. The RC should be a perfect insulator in the dark and should switch extremely rapidly in the light. A long-term extension would be to use an assembly like the RC as a molecular-scale, light-addressable wire between two electrode surfaces. Electrons are removed from the RC at the quinones, and a large variety of quinones can be substituted into the native binding site. By attaching a chain bearing a thiol to a quinone, it should be possible to connect the other end of the RC to an electrode as well. The approach we are taking should provide a more realistic strategy by using pre-assembled biological complexes as the key elements.

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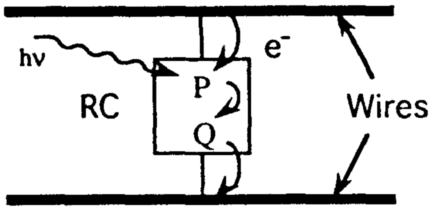

Figure 12. Schematic diagram illustrating the use of reaction centers as a light-driven high-speed switch between two conducting wires.

Chemical Sensors

Most biosensors fabricated to date depend on the indirect detection of the molecule of interest. In the system outlined above, the presence or absence of P^+ is directly detected by the electrode. The lifetime of P^+ depends on several factors. For example, if the quinone is reduced to Q^- or is absent, then the lifetime of P^+ is reduced to tens of nanoseconds (Figure 13). In this case, re-reduction of P^+ by the electrode is likely not to be possible, consequently no current will flow. Thus, current flowing into P^+ formed by light is a direct sensor of the state of the quinone. This leads to immediate applications of such a device to probe the ambient redox potential in the vicinity of the electrode based on the Q/Q^- couple. Because the quinone can readily be changed, the range of redox level over which the electrode is sensitive can be modified.

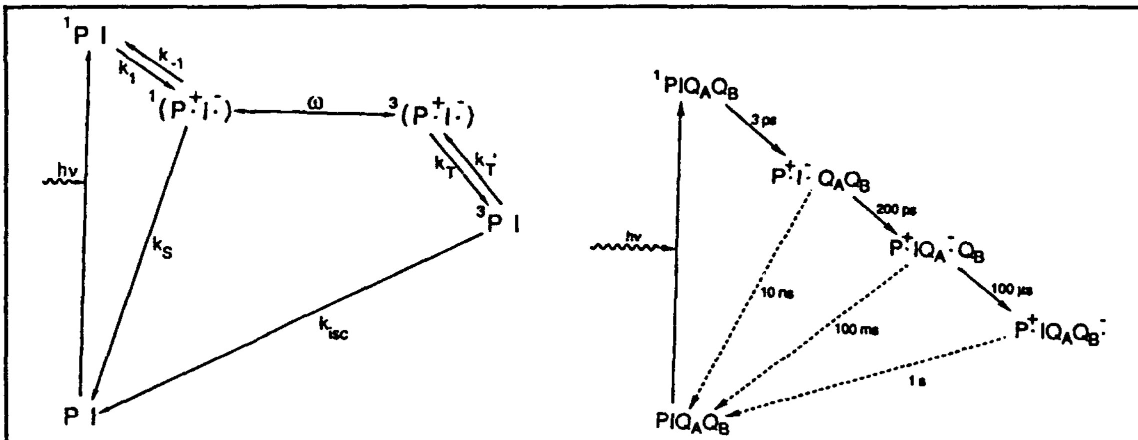

Figure 13. Reaction scheme for initial charge separation and recombination electron transfer steps in isolated RCs. This figure gives the known rates and approximate energies. The components are labeled according to the notation in Figure 3 where the intermediate acceptor I is H, the first quinone is Q_A and the second quinone acceptor Q_B is on the left side of the structure related to Q_A by the local C2 axis of symmetry.

A second interesting possibility is that replacement of the quinone by other, non-redox active, agents could be detected. It is well known that a number of herbicides replace quinones in RCs (this is one of several mechanisms by which triazine herbicides kill plants). Thus, an RC wired to an electrode could be used as a rather selective probe of the presence of herbicides in the environment. Many groups around the world are investigating modifications of the quinone binding site which may alter the binding of such herbicides (for example, fields containing plants which resist herbicides could be sprayed to eliminate all undesired weeds). This information can be turned around to

engineer quinone binding pockets which are highly selective for particular herbicides, potentially leading to inexpensive herbicide-selective biosensors based on RCs.

These examples illustrate very different applications, and in each case depend on developing the essential technology of covalently connecting the RC to a surface. The following section describes work with the RC which is applicable to the devices we have described.

Engineering the Reaction Center

CONTROLLING THE REDOX POTENTIAL OF THE SPECIAL PAIR. In order to further our understanding of the unidirectional (L-side only) charge separation steps, researchers in Boxer's group have changed a large number of amino acids which are in contact with P an are different on the right and left sides of Figure 1. These are called symmetry mutants because they increase the symmetry of the RC structure. The first of these, named Syml, makes most of the contacts with P into L-side amino acids (Woodbury, et al., 1990). Even though a large number of amino acid changes has been introduced (nine), these RCs assemble and can be readily isolated. Although we had hoped that electron transfer might now occur down the M-side branch of components, we found that it does not. This indicates, as mentioned above that the electron transfer is a function of the effective dielectric constant seen at the quinones. Instead there are relatively small changes in the rates of the charge separation steps. Surprisingly, these alterations in primary sequence substantially affect the mid-point potential for the P/P^+ couple, making it much more difficult to oxidize. This means that the driving force for the initial charge separation step is smaller than in wild-type.

Further examination of the changes in Syml suggested that a particular change, Phe(M)195 $\rightarrow$ His might introduce a hydrogen bond to the M-side BChl comprising P. This was tested by making the single amino acid mutation (M)F195H (see Figure 3, called the His/His mutant because symmetry-related (L)168 and (M) 195 are now both His), and, indeed, the special pair in this strain is as difficult to oxidize as in Syml. Woodbury and co-workers have also produced the reversed symmetry mutant (Phe/Phe) and find that P is easier to oxidize. Extensions of this work to many residues are currently in progress, and changes which affect the redox level of other components in the electron transport chain are in progress in a number of labs. The key point is that engineering of a critical aspect of RC function is possible. This basic result will likely prove to be important in the next generation of applications because it permits us to control the driving force for electron transfer between P^+ and externally appended redox components.

CONTROLLING THE RATE OF ELECTRON TRANSFER WITH EXTERNAL ELECTRIC FIELDS. As seen in the previous section, the redox potential for individual components can be shifted in discrete steps by changing amino acids in the environment of the components. An alternative and complementary approach is to change the free-energy level of charge-separated intermediates by application of an electric field. The interaction energy of a dipole with an electric field contributes to the free energy. For a charge-transfer process, the change in dipole movement is: $\Delta\mu = \mu_{final} - \mu_{initial}$, and the interaction energy with the external field, F_{ext}, is $\Delta U = -\Delta\mu F_{ext}$. Figure 14 illustrates why changing the energy of a dipolar intermediate will affect the rate of electron transfer. Figure 14 shows a typical calculated dependence of the rate of electron transfer on the free energy change for the reaction. By shifting the free energy upon application of an electric field it is possible to tune the rate of electronic transfer. In this sense, the applied field is analogous to the amino acid mutations which affect individual redox potentials discussed

in the last section. With the combination of shifts in potential produced by specific amino acid changes and external fields, it is possible to sample a wide range of driving forces.

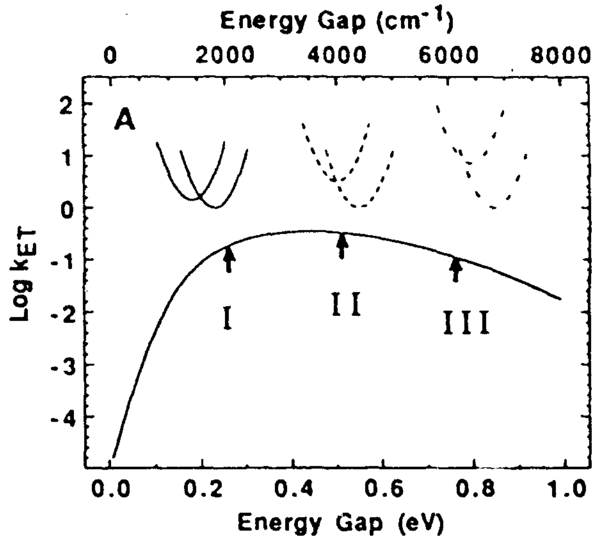

Figure 14. Schematic illustration of the origin of the effect of changing the driving force of an electron transfer reaction on the rate of electron transfer. I, II, and III are the normal, activationless (optimally exothermic) and inverted regions respectively.

During the past few years Boxer's group has investigated the effects of applied fields on the initial charge separation steps and on the long-distance, much slower $P^+Q_A^-$ charge recombination process. This is described in detail in the original papers Boxer, et al., 1989; Frazen, 1991). From a technological viewpoint these changes are not very interesting because the intrinsic rates (measured at low temperature) turn out to be relatively insensitive to applied field. Therefore, huge fields would be needed to change the populations of states in the reaction scheme to any appreciable extent. This is illustrated in Figure 15 for the $P^+Q_A^-$ recombination reaction, where it is seen that even at a very high applied field, the rate of recombination for a non-oriented sample is only weakly affected at low temperature. However, rather interesting results are obtained at room temperature because of the participation of competing, activated steps which can be greatly enhanced by the applied field. This is also shown in Figure 15, where it is seen that at room temperature the applied field causes a very large change in the $P^+Q_A^-$ decay kinetics. These plots are obtained by subtracting the decay measured in the absence of field from that obtained when the field is on during the decay (the field is off during the formation of $P^+Q_A^-$). Where the difference decay is positive, the rate of decay slows (e.g. at all times in the low temperature data); where the difference decay is negative the decay rate is accelerated by the field (e.g. at early times for the room temperature data). Because these changes in population are seen as large changes in absorption in the Q_Y band of P, this scenario represents a primitive electrical-to-optical switching device. At the present time the rare-limiting process is slewing the electric field (limited by the RC time-constant of the sample). This can likely be improved considerably by working with much smaller samples. Many possible variations on this theme can be imagined to produce very fast, short-term optical memories, special light modulators, and other electro-optical devices.

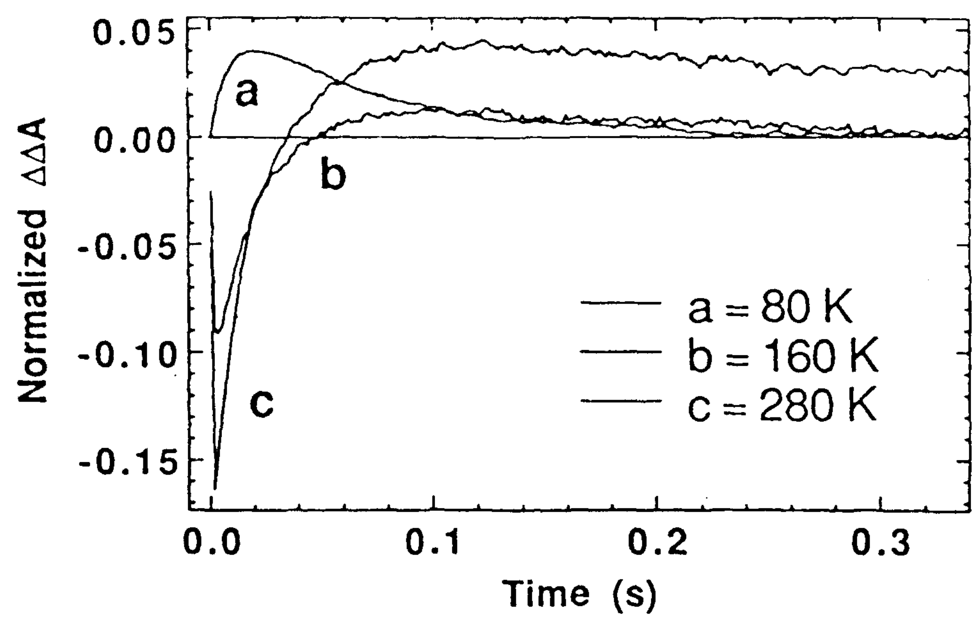

Figure 15. The effect of an applied electric field on the decay kinetics of the P^+Q_A state as a function of temperature. Where the normalized difference decay is positive, the rate is slower than in the absence of the field.

Preparation of Reaction Centers

The following outlines the main aspects of growth and large scale production of RCs.

Cell growth:

The photosynthetic bacteria are facultative, that is, they can grow photosynthetically in the absence of oxygen, or they can be grown fermentatively with a rich carbon source and oxygen in the dark. The latter is essential for the production of some mutants because RCs are produced but are not used by the organism, so there is no selective pressure to revert to wild-type. On the other hand, it is often possible to get a higher RC yield per liter of culture when the organisms are grown photosynthetically . The method used will depend on whether the mutations which are selected in Tasks I & II tend to revert or not. Both cases are described briefly in the following.

PHOTOSYNTHETIC GROWTH: wild-type organisms or stable mutants can be grown photosynthetically. Small bottles (50 mL) containing Gunnar's medium are inoculated with photosynthetic bacteria. The bottles are filled leaving no air space and the organisms are grown for 15 hours in darkness at 30°C. During this time, residual oxygen in the medium is consumed during fermentative growth. The bottles are then transferred to a light box and the organisms are grown for several days until cells begin to fall out of solution. At this point, the bottles are used to inoculate 500 mL or one liter bottles and the process is repeated indefinitely. This procedure gives the highest yield of RCs per liter of media. There are, however, problems scaling up photosynthetic growth and many

mutants are not stable to reversion, therefore, the following non-photosynthetic methodology has bee implemented.

NON-PHOTOSYNTHETIC GROWTH: For Rb. sphaeroides, cultures are removed from a slant or petri dish and are inoculated into sterile YCC media containing Casamino acids, yeast extract, and appropriate trace metal solutions. For Rb. capsulatus the growth medium is typically RCV+. Typically small cultures (5 mL) are grown to saturation at 34°C with vigorous shaking. After 24-48 hours, these cultures are used to inoculate larger cultures (typically 250 mL), the process is repeated, and the cultures are then scaled up to 3-4 liters. Alternatively, the 250 mL cultures can be used to inoculate an automated fermentor (typically 10 liters). Cells are oxygenated at a rate which just balances the consumption by the organisms. pH and temperature are also maintained by the fermentor. This process is readily automated and produces large yields of cells. Irrespective of production method, cells are harvested by centrifugation, typically using a Sharples industrial centrifuge for efficient pelletting of large volumes of culture.

Reaction center preparations:

RB. SPHAEROIDES: Cells are broken with a French pressure cell. Typically three passes through the cell give over 80% cell breakage. The broken cell suspension is then spun twice at about 10,000 g to remove unbroken cells. The membrane fragments left in suspension are called chromatophores. Chromatophores are pelletted by centrifugation at about 200,000 g, washed, and spun down again to ensure that all water-soluble proteins are removed. Reaction centers are then isolated by differential detergent extraction using variable percentages of lauryl dimethylamine oxide (LDAO). This is followed by final purification by ion-exchange chromatography. Typical media are DEAE-Sephacel with an ionic strength gradient provided by varying the salt concentration from 50 mM to 500 mM. Alternatively, the detergent solubilized RCs are banded on a sucrose gradient, followed by ion-exchange chromatography.

RB. CAPSULATUS: Chromatophore isolation is as above. LDAO is added to 1.5% to solubilize RCs and the mixture is added to a slurry of DEAE Sephacel. The RCs bind to the DEAE and they are pelleted by a brief spin. The DEAE with bound RCs is resuspended in buffer and poured onto a packed column of DEAE. The RCs are then eluted using a salt gradient.

More details on the preparation of reaction centers may be found in Appendices A and B. All of the steps in the growth of photosynthetic organisms and the preparation of reaction centers are documented there. Techniques for preparation of reaction centers have been transferred to BCC from Boxer's group at Stanford.

Stabilization of Reaction Centers

A common argument raised against the use of biological molecules in technological applications is that proteins are less stable than ordinary chemical compounds. While this is certainly true compared with some inorganic compounds, nearly all proposals for molecular electronic devices, including non-linear optical systems, require organic fragments. Proteins are no less stable than these molecules and offer many cost, flexibility and environmental advantages over complex organic molecules.

One of the main technical issues is degradation of the RC in the light due to production of a triplet state at the P site. A carotenoid molecule is contained in the natural reaction center in the M protein near the P site. This molecule quenches the triplet state of P and

prevents degradation of the reaction center due to sensitization of singlet oxygen formation *in vivo*. The effectiveness of this strategy for long term stabilization of the RC in vitro must be studied. Other alternatives include stabilization of the proteins by chemical treatment or other means. Many photosynthetic organisms live under quite extreme environmental conditions such as hot springs. The sequences of RC proteins from these organisms are beginning to become available. As has proven to be the case with proteins from other thermophillic organisms, it should be possible to determine some of the key stabilizing features which are present in these organisms and incorporate these into the RC primary sequence. For example, this strategy has been effectively developed by Genencor Inc. for producing improved forms of subtilisin which is widely used in laundry detergents. Other more direct routes to stabilizing the protein structure include the addition of surface cross-linking reagents such as gluteraldehyde. A range of these could be tested in order to enhance protein stability.

Conclusions

The major objective of this project was to develop methods for attaching photosynthetic reaction centers to a substrate. During the course of this project we identified an attachment site which would be highly advantageous for electron transfer from a conductive substrate to the reaction center. Subsequent experimental work demonstrated clearly that the residue at M189 could be substituted and a dye molecule attached at this site. This represents substantial progress toward the long range objective of demonstrating a working device based on the RC.

The other objectives of this project were to develop device concepts, to develop appropriate methods for preparation of reaction centers and to develop strategies for the stabilization of reaction centers.

> **Device concepts:** We have found exclusive of the materials issues related to deposition and stability, that reaction centers should be compatible with more conventional electronics. One very encouraging finding is that reaction centers, in combination with single electron transfer devices should make nanoscale photodetectors possible which are orders of magnitude more sensitive than those in use today.
> **Preparation of reaction centers:** We have documented all of the steps in the mutagenesis, preparation and analysis of photosynthetic organisms. A strain of *Rb. capsulatus* lacking the antenna complex is currently available. Using this strain to produce purified reaction centers will allow us to eliminate the extraction steps in the purification procedure which are associated with removing the antenna complex. This will simplify future device-related work.
> **Stabilization:** We have developed strategies for the stabilization of reaction centers. Improvement of reaction center stability may several investigations along different lines.

At the present time our consultant is proceeding with efforts to attach reaction centers to a substrate by means of covalent linkages. The need to preserve the reaction center in a lipid-like environment presents some technical difficulties; and once successful attachment is demonstrated, considerable work remains before electron transfer can also be demonstrated.

Technical Assessment

Potential Applications

Because the reaction center is a very efficient light to current transducer, we have found that the main applications of photosynthetic reaction centers should be in photosensors and light-driven chemical sensors. As discussed above, high-sensitivity nanoscale detectors are possible. Reaction centers have also been the target of considerable research into herbicides. Because many of these chemical agents interfere with the natural function of the reaction center itself, the RC could serve as a chemical detector for herbicides. The RC can be made selective by substituting various quinones for the native quinone. Further research into the modification of the RC proteins might also lead to modification of the chemical sensitivities of the reaction center. It would be desirable, of

course, to develop molecular structures which could detect and distinguish chemical agents other than herbicides.

Technical Issues for Phase II Program

The main technical objective for a Phase II program should be the deposition and attachment of RCs to a substrate by covalent linkages. The major technical issues are: (i) Choice and preparation of the substrate, (ii) Specific chemical groups to be attached to the RC in order to form the linkage with the substrate, (iii) Chemical processing necessary to stabilize the reaction centers without interfering with the attachment process, (iv) Demonstration of attachment of RC, (v) Demonstration of electron transfer, and (vi) Demonstration of a prototype photodetector.

Choice and preparation of substrate. We have discussed two alternatives, gold prepared with sulfhydryl groups and microporous films. These alternatives should be investigated to determine whether attachment is feasible and which type of substrate is most compatible with a photodetector design.
Specific chemical groups. Conjugated chains are preferable, to facilitate electron transfer. One problem to be resolved is the chain length to use in order that lipids or other molecules used to stabilize the RC will not block the attachment process. At the same time chain lengths should be short enough to permit efficient transfer.
Chemical processing. The specific chemistry to be used to form the covalent linkages remains to be worked out. We have discussed possible alternatives in the body of our report.
Demonstration of attachment. Specific experiments should be defined in order to conclusively demonstrate attachment. Observation molecular placement and electron transfer by an STM probe is one possibility, but additional experiments must be performed to eliminate the possibility of the type of artifacts which have plagued previous efforts to observe large organic molecules on conductive substrates.
Demonstration of electron transfer. Specific experiments designed to observe photo induced currents are also necessary. Again, one alternative is observation of current by means of an STM probe.
Demonstrate of a prototype detector. The major issue here is the compatibility of reaction center films with conventional microelectronics processing. The long term goal would be to demonstrate a nanoscale device.

Successful pursuit of a Phase II project, as outlined above would make a promising new material available to the scientific and engineering communities. It is the intention of BCC to pursue a long-term commitment to the development reaction centers as nanoscale components for electronics and photonics.

Acknowledgments

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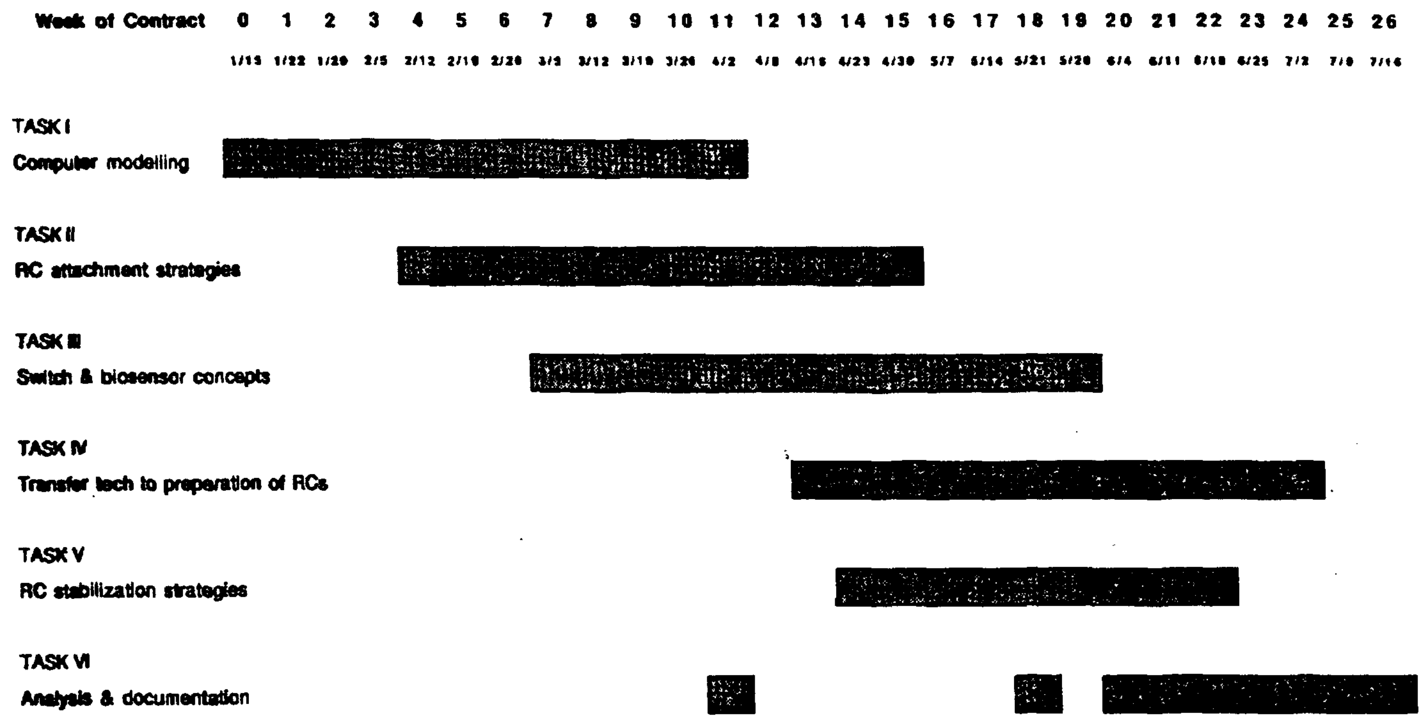
Milestone Chart



Appendix A: Procedures Used in the Mutagenesis, Preparation and Analysis of Capsulatus Reaction Centers

Introduction

The molecular biology of the photosynthetic bacteria is not substantially different from that of E. coli. In fact E. coli is a close relative of one branch of the evolutionary tree of these bacteria. At least some species are capable of conjugation with E. coli and of stably replicating the conjugation plasmid. They can grow either photosynthetically, aerobically or fermentatively if given a sink for electrons. Many of them are capable of nitrogen fixation as well. So far, the proper conditions for efficient transformation of these organisms has not been found, but conjugation between E. coli and at least one of the photosynthetic bugs, Rhodobacter capsulatus, is so efficient (several percent of the recipient bugs express plasmid genes) that for most purposes lack of transformation is not a hinderence. In addition, transfection using a viroid-like particle called a gene transfer agent (GTA) works very well for transfer and recombination of genetic material into the chromosome of capsulatus.

All of the genes asociated with the photosynthetic apparatus of capsulatus (and several other species) have been cloned and sequenced. These include the L, M, and H subunits of the reaction center, the alpha and beta subunits of B875 antenna complex as well as the subunits of the B800/850 antenna complex. The alpha and beta subunits of the B875 complex and the L and M subunits of the reaction center are organized into one operon. In addition to these genes the operon also encodes Q, a protein supposedly involved in the assembly of the protein subunits of the photosynthetic apparatus with the chlorophyll cofactors, and a couple of open reading frames of unknown function. The order of the genes is Q Alpha Beta L M ORF ORF.

We will only concern ourselves with this operon from here on in and will actually only discuss mutagenesis of the reaction center genes though similar work has been done on alpha and beta. Before one can transfer new reaction center genes into an organism, one has to have a strain that lacks the chromosomal copy of the operon. The strain obtained from Doug Youvan at MIT is called U43. It lacks the entire coding sequence for alpha, beta, L and M. In addition there is a point mutation in one of the B800/850 genes such that this antenna complex is also not expressed in these organisms. The original plasmid we obtained for transfering the operon from E. coli to capsulatus is called pU2922. This is based on the broad host range vector pRK290 and has the operon included in it as part of a linearized section of a pBR322 based plasmid called pU22

The plasmid pU2922 contains an origin of replication which functions in both E. coli and capsulatus as well as several tra genes for conjugal transfer. The rest of the genes required for transfer must be provided by the E. coli strain. For this reason S17 (also obtained from Youvan) is used as the E. coli strain pU2922 is transferred from. We have since modified pU2922 by removing several kb of sequence (including the AMP resistance) and several sites. The new plasmid is called pCR (plasmid for conjugation and recombination). This plasmid functions identically, as far as we know, to pU2922 (except for AMP resistance). It has the advantage of being somewhat smaller than pU2922 (and the name is much easier to say), and it has unique sites for both Bam and Xho I. These sites (particularly Bam HI) are useful for recombination work (see below).

This large vector should be used only when absolutely required because the transformation frequency is so low (about 100x lower). Therefore, most of the gene modification and subcloning is done in much smaller vectors. One that we use as an intermediate between the very small pUC-like vectors and pCR is called pU29. pU29 was developed by Youvan and is the same as pU22 described above, except that several sites have been removed and others inserted. In particular, it has unique Hind III, Bam HI, and KnpI sites which define the L and M genes.

The actual gene modification is done in smaller plasmids yet: subclones of pU29 in BlueScribe (a pUC-like vector from Stratagen) containing the Hind-Kpn fragment (L), the Kpn-Bam fragment (M) or the entire Hind-Bam fragment (L,M) of the reaction center genes. We refer to these as BS-L, BS-M and BS-LM respectively. These can be manipulated easily, have a number of convenient sites, and, more importantly, lack a number of unwanted sites. In the presence of a helper phage, single-stranded DNA copies of the inserts can also be produced for site-specific mutagenesis.

The general protocol is as follows:

Modify the gene of interest in one of the Bluescribe clones.

Transfer the Hind-Kpn, Hind-Bam, or Kpn-Bam fragment into pU29.

Transfer the EcoR1-SacI fragment of pU29 (which contains both L and M) into pCR or a derivative. (We often use pCR:0:lambdaES which is a pCR vector with nothing cloned into the Bam site, and a lambda phage DNA fragment replacing the reaction center genes between Eco and Sac. This way there can't possibly be any wild type plasmid contaminating the mutant plasmid.)

Below is a description of some of the methods involved in working with the plasmids and strains described above.

Cloning

If life were simple, all cloning could be done in pUC-like vectors, and as much as possible is. However, the biparental conjugation used to transfer genes into capsulatus requires the use of very large (25-30 kb) vectors which complicates matters significantly. So, in the descriptions that follow, procedures are given for both small vectors (pBS, pBR322, pU29 and derivatives) and for the larger vectors (pU2922, pU2922BX and derivatives). Any techniques not described here are done as indicated in Maniatus.

DH5 alpha cells from BRL are recommended. They are very competent (about 100,000,000/ug of Blue Scribe) and they have the additional advantage that when using plasmids which have LAC fused to their multiple cloning site (pUC, blue scribe, etc.) blue colony screening can be done without addition of IPTG to induce LAC. Only Xgal must be added. The only disadvantages of these cells are that they are rec+ and do not have the required background for conjugation.

When one can't use the frozen ones (suchas the S17 background for conjugation is needed -- S17 is rec- by the way) following is the alternative procedure recommended for making competent cells of S17.

Grow up 1 ml of S17 culture overnight from a glycerol stock (no antibiotic!)

Add 0.5 ml to 50 ml LB in 250 ml flask

Grow to mid log growth phase (for S17 this is about OD600 = .25, but it is essential to calibrate by doing a growth curve for any unknown bug). This usually takes 2-3 hours.

Ice for 10 min

Spin down in a 4 c rotor at 4000 rpm for 5 min (must be at 4 C before putting tubes in)

Resuspend in 25 mls of sterile, ice cold 50 mM CaCl2, 10 mM Tris pH 8

Ice for 15 min and then spin in 4 C rotor as above

Resuspend in 3 mls ice cold 50 mM CaCl2, 10 mM Tris pH 8

Aliquot into ice cold eppendorf tubes (about 0.5 ml/tube) and keep on ice for an additional 12-24 hrs (shorter times will decrease efficiency somewhat, but usually by only a factor of 2-3).

Assuming competent cells of either S17 or DH5 alpha, they can be used for a transformation. The transformation procedure itself is very similar for both cell types except that the volumes change. For S17 transformations with large plasmids we scale things up dramatically, since the efficiency is so low.

If DH5 alpha cells are used, remove them from the -70 C freezer and put them on ice for about 10 min to thaw. If it is a new batch, aliquot them into eppendorf tubes (50 ul/tube) and refreeze in dry ice ethanol those tubes not needed immediately. Store at -70.

Cool eppendorf tubes or clear plastic tubes (some claim these are better) down to ice temperature.

Set up a 42 C temp-block or water bath.

For DH5 alpha cells, take DNA from a ligation rxn and dilute it by a factor of 5 with H2O. Add 1 ul of each DNA to a cooled tube. Add 20 ul of cells.

Restriction

Using a restriction endonuclease amounts to diluting the appropriate premade buffer from the company, adding the DNA, and adding the enzyme. BRL enzymes come with tubes of appropriate buffers called React buffers. In any case the NEB catalog gives a table near the back of salt concentrations which work for different enzymes. It is worth noting that React 1 is OmM NaCl, React 2 is 50 mM NaCl, react 3 is 100 mM NaCl, and react 4 is 150 mM NaCl. They all contain a bit of Tris and MgCl2. 90% of all enzymes work in React 2. The NEB table will tell which ones don't. The only other buffer which is generally useful in this work is React 10 which is specially designed for Sal I. However, Sal I also works fine in React 4.

It is easiest up 1.25x solutions of these buffers ahead of time and then just add a total of about 2-3 microliters of DNA and enzyme to this. For example, for cutting minipreps to analyze on a gel:

1.25x buffer	8 ul
miniprep DNA	2 ul
enzyme	0.5 ul

incubate 2 hrs at appropriate temperature (almost always 37 C)

It is worth noting that most enzymes have a half life of about 1 hr, so digestion longer than 2 hrs is usually a waste of time. In fact, unless one are using a very expensive enzyme, 0.5 ul in a total of 10 ul will usually do the job nicely in 1 hr or less, For double digests (assuming the enzymes are compatible) just add 0.5 ul of each. Adding enzyme to a final volume of more than 10% is risky because the enzymes are stored in 50% glycerol and at glycerol concentrations greater than 5% the enzymes start doing odd things sometimes like cutting at new sites.

Although some researchers worry about dirty DNA interfering with restriction endonuclease activity, this is not a problem, largely because most enzymes are inexpensive enough and sold at high enough concentrations these days so that one can simply use more enzyme to overcome such problems. However, this can be a problem later for the transformation (see below).

Gel Purification of Fragments

It is sometimes necessary to cut restriction fragments out of agarose gels to be used later for a cloning experiment. There are at least two major ways of doing this. one involves using low melting point agarose, diluting the agarose (about x 5), phenol extracting (two or three times), and ethanol ppt. That works pretty well, but one may get higher yields using the electroelution method described below:

Cut out band from gel in a minimum amount of agarose.

Place this in a small size (about 1 cm diameter) dialysis tube with a minimum amount (usually 100-200 ul) of 0.5 x TBE (See Maniatus for preparation of dialysis tubing). Fill gel box with 0.5 x TBE. Place tube with gel piece perpendicular to long axis of the box and hold in place by putting something on top of it.

Electroelute for about 4 hrs at 100 V, then reverse the direction of current flow for 15 sec (the latter may be an old graduate student's tale, but I've been told that this helps remove DNA stuck to the wall of the dialysis tube).

Carefully remove liquid from the tube. Wash the gel piece and tube with another, say, 100 microliters of 0.5 x TBE. Total liquid should be less then 0.5 ml.

Phenol extract two or three times and ethanol precipitate.

Note: sulfates from the agarose inhibiting ligase reactions and transformations was once a concern. Presently agarose from most sources is pretty sulfate free (BioRad is good in this respect and FMC claims that it is possible to do the ligation IN their low melting point agar). For transformations of small vectors with reasonable amounts of DNA one may left out the phenol extractions and still had things work fine. For the large vectors, however, one need all the transformants one can get and the DNA should be made as clean as possible.

Ligations

Like restriction enzymes, ligase has become reasonably inexpensive and one can generally use enough so that reactions go to completion. When things don't work it is almost always because the DNA was lost somwhere along the line or because the transformation didn't work, rarely (a common occurence) because the ligation didn't work (assuming the ligase was not left out at room temperature overnight or something). It is recommended to do ligation reactions in about 20 microliters with about 100 ng (small plasmids) or 1-2 micrograms (large plasmids) of DNA.

For one reaction:

13 ul H_2O
2 ul 10x ligase Buffer (frozen at -20)
2 ul 10 mM rATP (frozen at -70)
2 ul DNA (this is about 100 ng for a miniprep or 1 ug for a maxiprep)
1 ul ligase (the lower conc. BRL ligase is fine for most work; for blunt end ligations use the higher conc. NEB ligase)
incubate at 16 C for 2 hr to o/n

Transformations

It is now possible to get quite a variety of frozen competent cells from BRL, Stratagen, etc. which generally work very well, and, if used wisely, are quite cost effective. For about $80 BRL will sell enough cells to do 50 simple, small vector ligation-transformations. For large vector transformations it is preferrable to transform into S17 (which they don't sell) so one have to make it for onself. The advantage of frozen competents is obviously that they are there whenever needed. It takes about a day to prepare competent cells (mostly waiting).

For S17 cells, take 2 ul of ligation mix and add to 18 ul of H2O in one of the tubes. In another put the remaining 18 ul of ligation mix. Add 0.5 ml of cells to each tube. With S17 we do this with and without dilution because which works best depends on the DNA prep. If neither DNA sample transforms, try cleaning up the DNA with a couple of phenol extractions. The junk in the DNA seems to often be the difference between getting 50 colonies and getting zero.

Ice for 30 min

Heat shock at 42 c for 2 min (S17) or 45 sec (DH5 alpha). Time it carefully.

Dilute by ten fold with LB (for DH5 alpha add about 200 ul; for S17 add 5 ml)

Let sit for 1 hr at 37 C with occasional gentle mixing.

For DH5 alpha, plate the whole 200 ul on one plate. For S17 spin cells down in clinical centrifuge and resuspend in 1 ml of LB. Plate on 5 plates (200 ul each).

What plates are used will depend on the plasmid. Obviously, the proper antibiotic must be present to select against nontransformed cells and in some cases Xgal will be desired for blue colony screening. Anything that takes longer than 24 hrs to come up will not be a good prep. BRL claims that the use of a medium comtaining glucose and Mg works better than plain LB during the 37 degree incubation before plating, but if so I have not noticed. It does not make it worse (see instructions that come with DH5 alpha cells).

Note that throughout the prep the phrase "ice cold" keeps appearing. This is extremely important. Except for the heat shock treatment keep them cold as long as they are in the 50 mM $CaCl_2$.

Boiling Miniprep

At this point one should have transformants and it is necessary to know what's there. There are several fast procedures for isolating small amounts of plasmids. Maniatus gives an alkaline lysis prep that normally used for maxipreps (see below) with slight modification. But, the miniprep procedure recommended is given below. It is fast and easy to do for large numbers of cultures at once and it is generally easy getting the resulting plasmids to cut with restriction endonucleases. This procedure is from Stratagen.

Pick colonies and grow up in 1ml of LB plus antibiotic in an eppendorf tube over night. (We have been just putting the eppendorf tubes in a beaker or flask in the shaker.)

Spin 5 min at 3000 rpm in microfuge

Make up enough STETL buffer for 110 ul per miniprep: Add lysozyme stock solution (the 15 mg/ml stock is stored in aliquots at -20 -- do not refreeze) to STET stock (stored at 4 C) to a final concentration of 0.5 mg/ml lysozyme.

Add 110 ul of STETL to each pellet

Resuspend and incubate at room temp for 10 min

Place in boiling water bath for 2 min

Ice for at least 1 min

Spin for 15 min in the microfuge in the cold room

Transfer the supernatant to fresh tubes

Add 110 ul of isopropanol, mix, and spin at room temp for 20 min at 13,000 rpm

Let pellets dry for a few minutes (they may not be visible) and then resuspend in 30 ul of TE.

At each stage in this prep where the pellet is saved, the supernatant is vigorously shaken out of the tube.

Alkaline Lysis Maxiprep

Assume the desired plasmid has been constructed, miniprepped and characterized. It may be necessary to grow more up before going on to the next step (this is often true if the next step involves the large plasmids for conjugation). The following procedure will accomplish that with a minimum of pain. This is basically straight out of Maniatus with a few modifications:

Grow up 100 ml of cells in LB + antibiotic (For pBR and other low copy number plasmids one can amplify as described in Maniatus, but it is easier to just grow more cells up if more are needed.).

Divide the culture into two of the 40 ml tubes for the small Sorval rotor (don't be greedy; just throw the rest out) and spin at 7000 rpm for 10 min.

Resuspend both pellets in a total of 5 ml of miniprep soln (the name comes from tradition) with 5 mg/ml lysozyme (add as a powder). Transfer everything into one tube.

Room temp for 5 min

Add 10 ml of 0.2N NaOH/1% SDS made fresh from stocks of NaOH and SDS. Invert tube gently several times. (It is preferrable to break open the cells without breaking the chromosomal gamish up too much.) Solution should become viscous.

Ice for 10 min

Add 7.5 ml 5M potassium acetate pH 4.8. Invert tube gently several times. (Here the chromosomal gamish should ppt and the plasmid should not. The efficiency of this separation depends on how badly the chromosome were damaged during this procedure and on how large the plasmid is.) Alot of white junk should fall out of solution.

Ice for 10 min

Spin at 15,000 rpm in small Sorval rotor for 20 min at 4 C

Pour supernatant into a fresh centrifuge tube. Add 12 ml isopropanol. Mix. Let stand at room temp for 15 min.

Spin in small rotor at room temp (roughly) for 30 min at 10,000 rpm

Pour off supernatant and rinse pellet with ice cold 80% ethanol to get rid of salt. Let pellet dry and dissolve in about 0.5 ml of TE

At this point one can either do a CsCl gradient (best) or a few phenol extractions followed by an ethanol precipitation (easiest by far). The latter is usually sufficient for most subsequent cloning procedures.

Bug Sex

Once construction is finished, one should have the mutant in pCR or some pCR derivative ready to transfer into capsulatus. The mating procedure follows:

Grow 2 ml of U43 in RCV+ to stagnation (starting from a single colony or from a glycerol stock this takes 2-3 days).

Grow 2ml of S17 with the plasmid (pCR or one of its derivatives with the desired modification in the RC genes) to late log phase (this takes about 8 hours but overnight usually works fine too).

Mix .7 ml of U43 with .3 ml of S17 and make five or six 50 ul spots of this on MPYE (NO ANTIBIOTIC). Allow 8-10 hours at 32 C (overnight also works).

Add 1ml of RCV to the plate and use a spreader to resuspend cells in this. Remove liquid. If a low frequency of conjugation is expected, spin the cells down (3-4 minutes at setting 3 or 4 on the microfuge) and then resuspend in RCV (in my experience this is usually not necessary).

Now do serial dilutions by factors of 100 down to 1/1000000 into RCV. Plate 200 ul of the original cells and each of the dilutions onto RCV plates with kanamycin. Typically, conjugates can be seen down to 1/10000. However, when we tried this with a construction in pU2922 which had a large repeated sequence (an M-M like construction) the frequency of conjugates that were kanamycin resistant was dramatically decreased. Only 7 colonies were recovered on the plate with no dilution.

After the cells grow up, pick a colony (or two) and streak out on RCV kanamycin. If there are still coli contaminants they are visible on the MPYE. Pick a single colony of capsulatus and grow up few mls in RCV+ and then prepare glycerol stocks (add glycerol to 15%, let sit at room temp 15 min, then put at -70 C).

Special Techniques

Recombination

One of the ways to produce mutants in the reaction center sequences of capsulatus is by recombination between those sequences and homologous sequences typically from related organisms. The vector developed for this is called pCR (plasmid for Conjugation and Recombination) and it has both the normal EcoRl Sacl cassette for the acceptor reaction center gene as well as both a BamHI and a XhoI site to put donor genes in. The acceptor gene is usually capsulatus reaction center sequences that have been rendered nonfunctional by some mutation. The donor genes are any homologous sequence that can be used to repair the mutation in the capsulatus operon. Since the acceptor genes usually contain a Bam site, the donor genes are put in first (donor gene insertion depends on there being only one Bam site in the plasmid if Bam is used for the donor).

Begin the construction with pCR:0:lambdaES which simply means a pCR vector that has nothing in the donor site and a small piece of lambda phage DNA as a space filler in the acceptor site. One will also need either a Bam-Bam or Xho-Xho fragment containing the donor and not containing any Eco or Sacl sites. This can often be done by taking a gene cloned into a multiple cloning site (MCS) of pUC or pBS, cutting out the whole site (Eco-Hind) and putting it into pBR322. Now by cutting with Bam one will get a Bam-Bam fragment that includes any insert in the MCS on the Hind side of the Bam site. Notice that both the Eco and Sac sites in the MCS have been left behind. Now clone this Bam Bam fragment into the BAM site of pCR:0:lambdaES producing pCR:donor:lambdaES. Select a clone which has the donor genes oriented pointing towards the Sacl site of the vector. In this way recombination between the donor and acceptor will produce inversions but no deletions and the plasmid will be more stable. Finally insert the Eco-Sac fragment containing the nonfunctional reaction center genes producing pCR:donor:acceptor. This transformation should be done in S17 or at least in some rec- strain. Now conjugate this into capsulatus and grow up 100 mls in RCV+ aerobically. Some fraction of this can be removed, spun down, resuspended in MPYE, diluted by a

factor with MPYE media containing succinate, kanamycin, and .75% agar that has been cooled to near gelling temp (about 35 C). The mixture is then poured onto a plate or similar device such that the liquid comes all the way to the top. The top of the plate should then be put on and be in direct contact with the agar, making the whole system anaerobic. This should be put in the light box at 30 C. Photosynthetic recombinants should appear in about 2-4 days. In the mean time, another 100 mls of culture can be innoculated and the process repeated after about 2 days. Happy hunting.

Analysis

Plasmid Isolation from Capsulatus

Perhaps the first thing to do with a potentially interesting mutant is to reisolate the plasmid from it and characterize it. The standard alkaline maxiprep on capsulatus grown in MPYE and Kan is recommended. DNA thus isolated was purified (lots of chromosomal contaminants) by transforming S17 and doing another mini or maxi prep out of that.

Chromataphore and Reaction Center Preparations

The preparation we use is based on one handed down from Doug Youvan with a few simplifying modifications. If one is going to go all the way to RCs, it is recommended to start with at least 1 liter of cells. For chromatophores, one may get enough for many purposes from 100 mls. The following instructions are are starting with a big 5 liter prep, but it is possible to scale it down.

> Grow cells in 4 liter flasks, 2.5 liter of RCV+ per flask. Shake slowly in the water bath at 30 C. If one starts with a 50 ml innoculum of aerobically grown cells the best results will be obtained by letting the cells go for 2-3 days. Spin down at 7000 rpm using the big Sorvall rotor. Pour off media into a flask carefully-the pellets are very loose. Don't just pour the spent media down the drain or the people downstairs get upset. Add bleach to the spent media to a final concentration of about 5% and let it sit in the hood for a few days..
>
> Resuspend in a total of 200 ml of Buffer A and French press at 20000 psi. Try to keep everything cold. These cells usually crack in one shot.
>
> Spin down cell debris and unbroken cells in the big rotor at 7000 rpm for 20 min. The pellet should be much less colored than before. If it is not that things did not break very well.
>
> Spin the supernatant in the Ti50.2 at 40000 rpm for 90 min.
>
> Pour off the supernatant from this run and resuspend pellet in about 20 ml of Buffer A (this completes the procecure for chromataphores) or buffer B (to on to reaction centers). Youvan calls for a second spin at high speed to get rid of some of the nonintegral membrane proteins but it doesn't seem to be necessary. It might be useful for cleaning up chromatophores before electrophoresis.

Adjust the optical density of the chromatophores to OD875=25 with buffer B. From here on in keep the light as dim as possible. Add LDAO (stock = 30%) to 1.5%, mix and incubate in a 37 C H_2O bath for 10 min. Place on ice.

Add about 1/3 (packed) volume of DEAE sephacell (Sigma and Pharmacia are fine) that has been equilibrated with buffer B and mix for 5 min. Spin at low speed (This can be done in four to six ml plastic tubes in the clinical centrifuge). Pour off supernatant and resuspend in buffer B. Repeat 4 more times. The DEAE should now be purple.

Resuspend the DEAE one more time and pour it on top of about 35 mls of packed DEAE in an approximately 1.5 cm diameter column that has had 2 column volumes of buffer C run through it and then 2 column volumes of buffer D. Wash the bound reaction centers with about 3 column volumes of buffer D. Initially some yellow stuff will come off then the eluate will turn clea. Now switch to Buffer B + 30 mM KCl. Run 1/2 column volume. Shift to buffer E. The RCs should start moving. Collect. A salt gradient might work better. A 30 mM to 300 mM KCl gradient in Buffer B has been recommended. The RC's obtained from this prep are purple (Youvan says green, but it depends on how much carotenoid is bound). One should get about 10 ODV (3 ODV = 1 mg) or more per liter of bugs. The ratios typically seen for the best fractions are 850:800:750:280 = 0.45:1.0:0.44:1.45. In the less good, but still acceptable (I think) fractions 0.43:1.0:0.44:1.7. The 850 peak should not be much above 1.7. The gradient may help to keep the 800:280 ratio lower.

Buffer A: 10 mM potassium phosphate, pH 7.35 made by diluting a 1M stock that was made up as the monobasic salt and titrated with potassium hydroxide.

Buffer B: A + 0.05% LDAO (remember stock LDAO is 30%)

Buffer C: B + 300 MM KCl

Buffer D: A + 0.6 % LDAO

Buffer E: B + 150 mM KCl

Protein Gels

Good results are difficult with protein gels run on capsulatus. Sphaeroides gives much cleaner gels particularly around the reaction center bands. The reaction center L, M, and H bands in chromatophore preps from capsulatus wild type and mutants that express these genes strongly. The following assumes that one has grown up cells in RCV+ semianaerobically (slow shaking at 30 C in the water bath) and isolated chromatophores. These should be resuspended to and OD875 of about 20. RCs are used as standards and are assumed for the following to be at an OD800 of about 10.

The general procedure for SDS PAGE follows:

Separating gel	5%	10%	15%
H2O	5.83ml	4.07ml	2.4ml
soln A	1.67ml	3.33ml	5.0ml
soln B	2.5ml	2.5ml	2.5ml
soln C	50.ul	50.ul	50.ul
10% APS	50.ul	50.ul	50.ul
TEMED	5.ul	5.ul	5.ul

soln A = 30% (w/v) acrylamide, 0.5% (w/v) bis
soln B = 1.5 M Tris pH 8.8
Soln C = 20% SDS
Soln D = 0.5 M Tris pH 6.8
sample buffer (2x) = 1ml of D, 2.5 ml of C (5% final conc), 1.5 ml Glycerol,
10 ul of 1% Bromophenol Blue
running buffer (5x) = 30 g Tris base, 144 g glycine, 5 g SDS
bring to 1 liter with H2O, pH to 8.3
10% APS = 10% (w/v) Ammonium persulfate

Assemble gel plates and spacers (how depends on apparatus). Mix up separating gel (I usually use 15% to look at reaction centers). Pour. Layer 400ul of H2O on top of acrylamide carefully using thin pipet tips from Stratagen. Let polymerize for about 1hr. Remove H2O and mix and pour stacking gel. Insert comb and let polymerize for about 1/2 hr. It is recommended to use as soon as possible after this to avoid excessive diffusion between stack and separating gels. Remove bottom spacer. Place on gel apparatus. Fill upper tray with running buffer. Check for leaks. Fill bottom tray. Carefully remove comb. Straighten and clean wells with running buffer. Attach electrodes. Load samples with thin pipet tips. Run at about 25 mA.

Samples are prepared by mixing 1:1 with 2x sample buffer and incubating at 70 C for 5 min. For reaction center samples it is recommended to dilute them to OD800=2 before mixing with sample buffer and load 5 ul. For chromatophores one can use them straight at OD875=20 (mixed 1:1 with sample buffer giving a final conc. of 1/2 that) and load 5-20 ul. Notice the high SDS conc. in the sample buffer (1x sample buffer has 5% SDS). This is required to properly solubilize the RCs. If one uses much lower concentrations one only see H.

After the run disassemble apparatus and place the gel on a shaker in Coomassie stain soln (0.1% Coomassie Blue, 40% MeOH, 10% Acetic Acid) for about 1 hr to overnight depending on gel size etc. (2 hrs usually does it). Place gel in destain soln (40% MeOH, 10% Acetic Acid) on a shaker and check every hr or so until destaining is complete. When complete, soak in 10% glycerol for about 30-60 min and then dry on gel dryer.

Growth Media

LB

This is for growth of E. coli. Per liter: 10 g bacto tryptone, 10 g NaCl, 5 g yeast extract, autoclave. For plates add 15 g bacto agar. Cool below 60 C before adding antibiotics.

MYPE

This is a general purpose complex media for rapid growth of either capsulatus or coli. It is not selective. It can also be used for photosynthetic growth of capsulatus (see below).

	5 l	1 l	500 ml	100ml
Peptone	15 g	3 g	1.5 g	0.3 g
Yeast Extract	15 g	3 g	1.5 g	0.3 g
1M $MgCl_2$	8 ml	1.6 ml	0.8 ml	0.16 ml
1M $CaCl_2$	5 ml	1 ml	0.5 ml	0.1 ml
To boost photosynthetic growth add:				
15% Succinate	60 ml	12 ml	6.0 ml	1.2 ml
For plates add:				
Bacto Agar	45 g	15 g	7.5 g	1.5 g

Autoclave and serve. Let cool to about 50 C before adding Kanamycin or other similar condiments.

RCV

This is a defined medium for capsulatus which does not support coli. This medium is used any time one wishes to grow up capsulatus specifically in the presence of potential contaminants. Note that growth on RCV is about half as fast as on MPYE under aerobic conditions. Also the author has observed that on plates, at least, if one restreaks repeatedly from one RCV plate to the next, growth becomes more and more feeble. It is possible the water used in the preparations is too pure and some essential contaminant is missing.

	5 l	2.5 l	1 l	500 ml	100 ml
H2O	4425 ml	2212 ml	885 ml	442 ml	88.5 ml
10% $(NH_4)_2 \cdot SO_4$	50 ml	25 ml	10 ml	5 ml	1 ml
10% DL Malic Acid-6.8	200 ml	100 ml	40 ml	20 ml	4 ml
Super Salts	250 ml	125 ml	50 ml	25 ml	5 ml
0.64M KPO_4-6.8	75 ml	37.5 ml	15 ml	7.5 ml	1.5 ml

Add water first, KPO_4 last. Autoclave (usually a little precipitate but not too much). Cool to 50 C before adding antibiotics. For plates make above media up at double strength:

	5 l	2.5 l	1 l	500 ml	100 ml
H_2O	1875 ml	938 ml	375 ml	187 ml	37.5 ml
All Else		AS BEFORE			

and make up an equal volume of 3% Bacto Agar:

	5 l	2.5 l	1 l	500 ml	100 ml
Bacto Agar	75 g	37.5 g	15 g	7.5 g	1.5 g
H_2O	2.1 l	1.25 l	500 ml	250 ml	100 ml

Autoclave the two solutions separately and then mix.

RCV+

This is a supplemented version of RCV made for anaerobic (fermentative) growth of capsulatus. It contains DMSO as an electron sink. Use this media to grow mutants on when it is desired to express reaction centers and antenna (which happens under anaerobic conditions), but not required that the reaction centers and antenna function photosynthetically.

	5 l	2.5 l	1 l	500 ml	100 ml
RCV (autoclaved)	5 l	2.5 l	1 l	500 ml	100 ml
glucose/pyruvate	200 ml	100 ml	40 ml	20 ml	4 ml
DMSO	18 ml	9 ml	3.6 ml	1.8 ml	3.6 ml

DON'T autoclave this. The glucose/pyruvate solution is made by adding 30 g glucose and 25 g pyruvate to a beaker and bringing the H_2O level up to 200 mls. Next filter sterilize through .4 micron filter (use a big syringe and one of the syringe adapting filters). DMSO does not require sterilization.

10% DL Na Malate pH 6.8

This is needed for RCV. For 1 l:
100 g DL Malic Acid
60 g NaOH (be accurate-not too much!)
bring H_2O to 1 l

The pH may be a little low at this point. If so make up 1 N NaOH and add slowly. Remember this is well above the pK of Malic Acid.

Super Salts

This is needed for RCV. For 1 l:

0.4 g 1% disodium EDTA
4 g $MgSO_4 \cdot 7H_2O$
1.5 g $CaCl_2 \cdot 2H_2O$
20 ml Trace Elements (see below)
.24 g $FeSO_4.7H_2O$
20 mg thiamine

Trace Elements

This is needed for RCV. For 250 ml:

0.397 g $MnSO_4.H_2O$
0.700 g $H3BO_3$ (boric acid)
0.010 g $Cu(NO_3)_2 \cdot 3H_2O$ (cupric nitrate)
0.060 g $ZnSO_4.7H_2O$
0.187 g $NaMoO_4.2H_2O$ (sodium molybdic acid)

Antibiotics

For coli growth:

Geopin (stable form of AMP) 50-100 ug/ml
Tetracycline 15 ug/ml
Other antibiotics see Maniatus

For capsulatus growth:

Kanamycin 10-15 ug/ml

Buffers and Solutions

TE

10 mM Tris, 1 mM EDTA diluted from a 10x stock which is made from Tris base and titrated to pH 8.0

TBE

1x = 0.089M Tris-borate, 0.089M boric acid, 2mM EDTA

To make up 5x:
54 g Tris base
27.5 g Boric acid
20 ml 0.5 M EDTA (pH 8.0)
H2O to 1 liter, pH should be 7.5-7.8

Ligase buffer

IN BRL CATALOG

Phenol/Chloroform for Phenol extractions

It is recommended to buy redistilled phenol for small lots. Equilibrate it with 1M Tris pH 8.0 by extracting until the pH of the aqeous phase no longer changes. Then equilibrate with 10 mM Tris pH 8.0. Add an equal volume of chloroform.

Miniprep solution (for maxipreps)

50 mM glucose, 10 mM EDTA, 25 mM Tris pH 8.0

STET (for minipreps)

7% sucrose, 0.5% Triton X-100, 50 mM EDTA pH 8.0, 10 mM Tris pH 8.0

5M potassium acetate pH 4.8

To 60 ml of 5M potassium acetate add 11.5 ml Acetic Acid and 28.5 ml H2O. The resulting solution should be 3 M in K and 5 M in Ac. The pH should be about 4.8.

Appendix B: Procedure for Growing Large-Batch Cultures of R. sphaeroides

Materials

1. Slant or petri plate containing desired bacterial strain
2. Sterile 50 ml steel-capped culture tubes
3. Clean 250 (or 500) ml steel-capped Erlenmeyer flasks
4. Innoculating loop
5. Sterile 5 ml pipettes
6. Clean 4 liter Erlenmeyer flasks with appropriate stoppers
7. Shaker with rack for 50 ml culture tubes and clamps for 250 ml and 4 liter flasks rotating at about 200 rpm
8. Antibiotic specific for desired strain in appropriate concentration:
   for R. sphaeroides requiring Tetracycline a solution with a concentration of 3 mg/ml should be made up so that 1 microliter of the solution per ml of medium is used
9. YCC Medium: per liter

Casamino Acids	6 g
Yeast Extract	5 g
Solution C	5 ml

adjust pH to 7.2 with KOH or NaOH (5 M or 1 M depending on volume)

10. Solution C: per liter

Nitrilotriacetic acid	10.0 g
$MgCl_2 \cdot 6H_2O$	24.0 g (or 11.4 g MgCl2 anhydrous)
$CaCl_2 \cdot 2H_2O$	3.335 g
Metals "44"	50 ml

Dissolve NTA in about 500 ml water and 7.3 g KOH; add rest of ingredients and neutralize to pH=6.7-6.8 with 5 M and 1 M KOH (about 8.5 ml of 5 M is required per liter). Allow precipitate to dissolve between additions of KOH.

Methods

Day 1 (1.) Using sterile technique, pipette 5.0 ml of sterile, liquid YCC medium into a sterile 50 ml culture tube; add about 1/2 loopful of culture from a slant or a single colony from a plate using a flamed innoculating loop; add 5.0 microliters of a 3 mg per ml Tetracycline solution

Day 1 (2.) Grow 5 ml culture to saturation (24 to 48 hours) at 34 degrees C on a shaker

Day 1 (3.) Fill 250 ml Erlenmeyer flask with 200 ml of YCC medium; fill 4 liter flask with 3.5 liters of YCC; autoclave

Day 2 or 3 (4.) Sterilly transfer (pour) the saturated 5 ml culture into a 250 ml flask filled with 200 ml of sterile YCC; add 200 microliters of a 3 mg/ml Tetracycline solution

Day 2 or 3 (5.) Grow 200 ml culture to saturation (24 to 48 hours) at 34 degrees C on a shaker

Day 3 or 4 (6.) Using sterile technique, pour the saturated 200 ml culture into a 4 liter flask filled with 3.5 liters of sterile YCC; add NO antibiotic

Day 3 or 4 (7.) Grow 3.5 liter culture to saturation on a shaker for 4 to 5 days; harvest cells

Note: This is be one of the most time-efficient methods, however, if desired, 35 ml of sterile YCC can be added to the saturated 5 ml culture, and when this 40 ml culture is saturated, it can be poured into either 200 or 300 ml of sterile YCC. This culture, in turn, can be poured into 3.5 liters of sterile YCC when it is saturated.

Solutions for RC Prep

Solutions should be pH'd before adding LDAO.

Buffer 1:	1mM EDTA	YCC medium:	casamino acids	6g/L
	15 mM Tris		yeast extract	5g/L
	100 mM NaCl		solution C	5ml/L
	(pH 8.0)		(pH 7.2 w/KOH or NaOH)	
Buffer 2:	1 mM EDTA	Solution C:	nitriloacetic acid	10g/L
	10 mM Tris		$MgCl_2 \cdot 6H_2O$	24.0g/L
	0.1% LDAO		or $MgCl_2$	11.4g/L
	(pH 8.0 w/HCl)		$CaCl_2 \cdot 2H_2O$	3.335g/L
			metals 44 sln.	50ml/L
Buffer 3:	1mM EDTA	Metals 44:	EDTA	2.5g/L
	15 mM Tris		$ZnCl_2$	5.22g/L
	100mM NaCl		$FeCl_2 \cdot 4H_20$	5.02g/L
	0.1% LDAO		$(NH_4)_6Mo_7O_{24} \cdot 4H_20$	0.185g/L
	(pH 8.0)		$CuCl_2 \cdot 2H_2O$	0.156g/L
			$Co(NO_3)_2 \cdot 6H_2O$	0.248g/L
			boric acid	0.144g/L
			$MnCl_2 \cdot 4H_20$	1.8g/L

Rb. Spheriodes Prep.

Bug Growth:

1) grow strains in sterile YCC medium, foil-covered flasks shaken vigorously at 34 C. (NOTE: For WT, do not add tetracycline)
    - a) stab of frozen culture into 5 ml YCC + 5 ul tetracycline, 2 days
    - b) dilute into 200 ml YCC + 200 ul tet., 2 days
    - c) dilute into 5.2 L YCC (no tet.), 6 days

2) harvest cells on Sharples T-1 contiuous flow centrifuge, 8ml/s. (Harvest varies 20-80 g cells per 20 L culture)

3) spin cells and recovered medium 8K rpm, 20 min. in Sorvall to remove excess YCC.

4) slurry pellet in minimum amount Buffer 1, spin again.
5) freeze pellet -80 C

French Press:
1) slurry pellet 2:1 with Buffer 1.
2) press 2-3x at 18,000-20,000 psi, changing ball often.
3) spin 8k rpm, 20 min. in Sorvall. Save supernatant (RCs +), store pellet for next prep.

Isolation of RCs:

1) LDAO wash #1
a) dilute press supernatant to 50 OD at 850 nm in 1 cm cell.
b) *slowly* add LDAO to 0.25% (w/v), stir 15 min. Keep sample dark.
c) spin in Quick Seal tubes 50k rpm, 1.5 hrs., 4 C in 50.2 Ti rotor.
d) snip off top of tubes, squirt out straw-colored supernatant. Check spectrum of supernatant for RCs (if colored), save pellets (RCs +).

2) Homogenize pellets with minimum anount of Buffer 1.

3) LDAO wash #2
a) dilute slurry to initial volume of wash #1 in Buffer 2
b) slowly add LDAO to 0.50% (w/v) stir 15 min.
c) spin as above in 50.2 Ti rotor.
d) save supernatant (RCs +) and take spectrum. Resuspend pellets and check spectrum for RCs.

4) $(NH_4)_2SO_4$ (a.s.) Precipitation #1
a) add a.s. stock 1:3 parts supernatant. (ca. 12.5% $(NH_4)_2SO_4$)
b) spin 8k rpm, 30 min. in Sorvall.
c) decant supernatant (RCs +), resuspend pellet

5) $(NH_4)_2SO_4$ (a.s.) Precipitation #2
a) add 2x volume a.s. stock as precipitation #1.
b) spin as above in Sorvall.
c) *gently* remove centrifuge bottles from rotor. Levitate is RCs. Remove all supernatant (should be mostly LH2), as $(NH_4)_2SO_4$ ruins sucrose gradient, check for RCs. (NOTE: if only partially precipitated, dilute total volume 2x with a.s. stock. Dialyze RCs after step e)
d) resuspend levitate in 4 mls Buffer 2 per Quick Seal tube used in LDAO steps. Do not exceed 60 mls.
e) spin 8k rpm, 20 min. in Sorvall. Save supernatant (RCs).
f) If not all of the $(NH_4)_2SO_4$ removed in step c then the RCs should be dialyzed 24 hrs in Buffer 2.

6) Sucrose gradient
a) fill Quick Seal tubes with 20 ml 35% sucrose in Buffer 3. Freeze tubes -80 C for 1 hour. Layer on 20 ml 7% sucrose solution and thaw tubes in vertical position. (ca. 2 hrs)
b) lay tubes on side for 8 hrs at RT.
c) reposition tubes to vertical and remove 5 ml from top.

d) delicately layer on 5 ml RCs per tube with blunt-tip syringe.
e) spin 50k rpm, 4 C, in 50.2 Ti rotor, 9 hrs (or longer, but do not let tubes sit after spin).
f) snip off top of tube. Remove layers of supernatant from top down. Check spectrum of all bands. RCs should be 1st purple band from top.

7) Chromatography column
a) column material is DEAE sepharose CL-6B, equilibrated with Buffer 2 for at least 2 hrs. in cold room, 3 ml/min.
b) pool fractions containing RCs, dilute 1:1 in Buffer 2.
c) load RCs onto column, wash with Buffer 2, 1 hr.
d) elute with NaCl gradient in Buffer 2. (100 mM to 350 mM: 150 mL each) 1ml/min. Band's off are RCs (1st, ca. 2 hrs.) and LHII antennae (2nd).
e) pool all bands with RCs, desalt in Amicon cell with PM-30 membrane to final NaCl<100 mM.

8) FPLC column
a) column material is Toyopearl DEAE 650S, packed with 1M NaCl in Buffer 2 under positive pressure, 3 ml/min.
b) place desalted, concentrated RCs in S&S spin filter (0.45µ)
c) add LDAO to side of tube to final conc. of 1%
d) spin 5-10 min in the dark, store for 15 min at 4 C.
e) inject RCs onto column at 0.5 ml/min. rate, equilibrate to 30min.
f) linear gradient: 0->50% 1M NaCl in 30 minutes, 3 ml/min. RCs off 1st, should be>90% pure.

Storage

1) aliquot RCs into screw-cap eppendorfs.
2) freeze -80 C, no glycerol.